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REGULATION OF OXYTOCIN RECEPTOR EXPRESSION BY ACTIVATOR PROTEIN-1 IN HUMAN MYOMETRIAL CELLS

by

Allison Laura Ball

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Medical Sciences - Obstetrics and Gynecology

Edmonton, Alberta

Fall 2003

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Aug 5/03

Date Submitted

ABSTRACT

The concentration of the oxytocin receptor (OTR) increases in the myometrium at term in human pregnancy. However the mechanism by which this occurs is unclear. The regulatory region of the human OTR gene contains two binding sites for the transcription factor Activator Protein-1 (AP-1). Therefore, we hypothesized that AP-1 regulates expression of the OTR gene in human myometrial cells in vitro. After stimulating AP-1 using TPA, OTR mRNA was found to increase after 24 hours while OT binding increased after 1 hour but not at 24. This suggests both a genomic and non-genomic effect of PKC-mediated signal transduction pathways on OTR regulation. Adenoviral overexpression of the AP-1 proteins however, did not induce OTR mRNA, suggesting that, contrary to our hypothesis, AP-1 does not appear to be the principle factor involved in PKC-mediated OTR transcription. Understanding the regulation of OTR at both the genomic and non-genomic level may lead to an improved understanding of the mechanisms involved in the initiation and progression of labour.

ACKNOWLEDGMENTS

Firstly, I would like to thank my supervisor Dr. Peter Mitchell. He promised me that I would come out of this experience "a better woman" and I am beginning to believe that he is right. His cheerfulness and encouragement has made the past two years of working with him a complete joy. His support has often extended from the academic to the personal, and for being an excellent mentor, I thank him.

Thank you to my supervisory committee members, Dr. Larry Guilbert and Dr. Sandra Davidge. They have also been nothing but encouraging and positive throughout my studies.

To Susan Wong, Dean Zaragoza, and Dr. Xin Fang, thank you for your patience and phenomenal technical support! To Sheila McManus and Eileen Marco, thanks for all the administrative support and the work you have done for me over the past two years.

****

This thesis is dedicated to my family, without whose support I would not have devoted myself to the pursuit of academic excellence and personal growth. Mom, Dad, Michelle, Kelly and Jaxon, thank you for being my best cheerleaders - for kicking me in pants when I needed it most and for telling me that I could achieve anything I put my mind to. You have all been an unfailing source of support, wisdom, and encouragement. I feel that you have been on this journey with me.

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ABBREVIATIONS AND UNITS

The following abbreviations, definitions and units have been used throughout this thesis.

°C .....degrees Celsius

AP-1 .....activator protein-1

ATF .....activating transcription factor (a.k.a. CREB)

ATP .....adenosine triphosphate

BSA.....bovine serum albumin

bp.....base pair

Ca^{2+} .....calcium ion

$CaCl_2$.....calcium chloride

CaM IIK.....calmodulin II kinase

cAMP .....cyclic adenosine monophosphate

CBP.....CREB binding protein

CK-I .....casein kinase I

CRE.....cAMP response element

CREB .....cAMP response element binding protein (a.k.a. ATF)

cGMP .....cyclic guanidine monophosphate

CKII.....casein kinase II

CO_2.....carbon dioxide

DMEM .....Dulbecco's modified eagles medium

DMSO .....dimethylsulfoxide

dNTP .....deoxy-nucleotide triphosphates

DAG .....diacylglycerol

DTT .....dithiothreitol

E_2.....estrogen

EDTA .....ethylenediaminetetraacetic acid di-sodium salt

EGTA .....Ethylene-bis(oxyethylenenitrilo)tetraacetic acid

ELISA .....enzyme-linked immuno assay

EMSA .....electrophoretic mobility shift assay

ERK.....extracellular-signal-regulated protein kinase

et al.....*et alii* (Latin, 'and others')

EtBr .....ethidium bromide

EtOH .....ethanol

FBS .....fetal bovine serum

g.....acceleration due to Earth's gravity (9.8 $m \cdot s^{-2}$)

g.....gram(s)

GFP .....green fluorescent protein

GRK.....G-protein related kinase

GSK.....glycogen synthase kinase

H_2O .....water

HCl.....hydrochloric acid

HEPES .....4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid

HPRT .....hypoxanthine ribosyltransferase

i.e......*id est* (Latin, 'that is')

IL1β.....interleukin-1β

IP3 .....inositol trisphosphate

JNK .....cJun amino terminal kinase

kB.....kilobases

KCl.....potassium chloride

kD.....kilodalton

l .....litre(s)

m .....meter(s)

M.....moles·l^{-1}

$MgCl_2$.....magnesium chloride

MAPK.....mitogen activated protein kinase

min .....minute(s)

MLC.....myosin light chain

MLCK.....myosin light chain kinase

MLCP.....myosin light chain phosphatase

MOI.....multiplicity of infection

MOPS.....morpholinepropanesulfonic acid

mRNA.....messenger ribonucleic acid

MTT .....3-(4,5-dimethylthioazol-2-yl)-2,5-diphenyltetrazolium bromide

n.....number of experiments

N.....normal

NaCl .....sodium chloride

NaN_3.....sodium azide

NFκB.....nuclear factor kappa B

N-terminal .....amino-terminal

O_2 .....molecular oxygen

OD.....optical density

OT .....oxytocin

OTR.....oxytocin receptor

p value.....probability (of incorrectly rejecting the null hypothesis)

P_4.....progesterone

PBS .....phosphate buffered saline

PCR.....polymerase chain reaction

pH.....power of hydrogen (logarithmic unit measuring acidity)

PIP_2 .....phosphotidylinostiol trisphosphate

PG .....prostaglandin

PGHS2 .....prostaglandin H synthase 2

PKA.....protein kinase A

PKC.....protein kinase C

PKG.....protein kinase G

PLCβ.....phospholipase C beta

PMSF.....phenylmethylsulfonylfluoride

RNA.....ribonucleic acid

RT.....reverse transcription

SDS.....sodium dodecyl sulphate

SEM.....standard error of the mean

TPBS.....phosphate buffered saline with Tween

TE.....Tris-EDTA

TNFα.....tumor necrosis factor alpha

TPA (PMA).....12-O-Tetradecanoylphorbol 13-acetate (Phorbol 12-myristate 13-acetate)

Tris-Cl.....tris[hydroxymethyl]-amino methane hydrochloride

TRE.....TPA response element

tTA.....tetracycline-responsive transactivator

ULTR.....uterine muscle cell line

<u>Mathematical prefixes</u>

k.....kilo (10^3)

c.....centi (10^{-1})

m.....milli (10^{-3})

μ.....micro (10^{-6})

n.....nano (10^{-9})

Amino Acid Abbreviations

Amino Acid			Amino Acid		
Alanine	Ala	A	Leucine	Leu	L
Arginine	Arg	R	Lysine	Lys	K
Asparagine	Asn	N	Methionine	Met	M
Aspartic Acid	Asp	D	Phenylalanine	Phe	F
Cysteine	Cys	C	Proline	Pro	P
Glutamic Acid	Glu	E	Serine	Ser	S
Glutamine	Gln	Q	Threonine	Thr	T
Glycine	Gly	G	Tryptophan	Trp	W
Histidine	His	H	Tyrosine	Tyr	Y
Isoleucine	Ile	I	Valine	Val	V

CHAPTER 1. INTRODUCTION, BACKGROUND, AND HYPOTHESIS.

1.1 THE CLINICAL PROBLEM OF PRETERM LABOUR

The initiation and progression of labour are fields of study in which little is known. Researchers and clinicians alike seek to understand the mechanisms of parturition in order to manage the preterm labouring patient to improve both fetal and maternal outcome. According to the World Health Organization, birth is considered preterm if it occurs before 37 weeks gestation.[1] The incidence of preterm birth in Canada is approximately 7.5% and despite years of research, it continues to increase steadily[2,3] (Figure 1). Approximately 75% of neonatal deaths occur in babies that are born prematurely, and despite improved survival rates of premature and low birth weight babies in recent years, there has been a concurrent increase in short- and long-term morbidity.[2] Premature infants often need long-term neonatal care, and recent evidence shows that low birth weight (usually as a result of preterm delivery) is a contributing factor to many adult-onset diseases such as coronary heart disease, hypertension, and type II diabetes.[4] Further, notwithstanding the emotional toll, there is a great economic burden resulting from preterm birth and its effects. In Canada, a recent figure estimates the cost per year of caring for preterm infants and the long-term care that results, to be approximately $13 billion dollars.[5]

Preterm birth can occur for several reasons and these have often been categorized accordingly. An example of such categorization is seen in Table 1. These designations are proposed to better understand the causes of preterm birth and to potentially tailor its treatment. However they must be regarded as only a guideline, for a large proportion of preterm cases are idiopathic. Further, a pertinent question remains: is preterm labour a physiological process that simply occurs too soon, or is it a pathophysiologic process in

and of itself?[6] Without a clear answer, we run the risk of simplifying a complex multi-factorial disorder, the etiology of which has yet to be determined.

As the causes of preterm birth are diverse and often unknown, its treatment is often generalized and non-specific. This is an unfortunate reality of clinical treatment over the past fifty-odd years. The term "tocolysis," was introduced in 1964 after the identification of an emerging interest in delaying premature labour contractions. Early tocolytic agents included relaxin and ethanol, while current therapies include one or a combination of magnesium sulphate, beta-mimetics, oxytocin antagonists, calcium channel inhibitors, adrenergic beta-receptor agonists, nonsteroidal anti-inflammatory drugs, and prostaglandin synthase inhibitors.[7] These agents target the contractile machinery in the uterus and work with varying degrees of effectiveness. The use of such tocolytics may delay labour for only up to 48 hours. However, that does provide time for transfer of a patient with the fetus *in utero* or to administer corticosteroids, which is associated with a decrease in fetal respiratory distress syndrome and mortality.[8] In general however, tocolytics have not been shown to provide significant improvement in neonatal outcome and are often associated with adverse effects.[1,8] In addition, research has shown that the preterm birth rate has not declined despite the development and use of tocolytics. In fact, Goldenberg and Rouse state that "most interventions designed to prevent preterm birth do not work, and the few that do…are not universally effective and are applicable to only a small percentage of women at risk for preterm birth."[8] The unfortunate reality is, that even after much research and increased understanding of the contractile mechanisms of the uterus, there is still not an effective tocolytic agent on the market to delay labour a significant amount of time.[10]

Continued research in the field of preterm birth has led to a greater understanding of the cellular events associated with onset and progression of labour, but has failed to give us a clear picture of the steps directly leading to uterine contractions in both the term and preterm labouring patient. Clearly, the study of the physiology and pathophysiology of labour onset is of vital importance.

1.2 UTERINE ACTIVATION

1.2.1 Uterine activation and the contraction associated proteins

Nearing the end of the third trimester, the uterus prepares for the onset of labour. This preparatory phase is termed "activation" and is associated with the upregulation of a group of genes known as the contraction associated proteins (or CAP genes). Examples of CAP genes include: connexin-43 (Cx-43), the major gap junction protein, prostaglandin H_2 synthase (PGHS2), an enzyme which catalyses the formation of prostaglandins, prostaglandin $F_{2\alpha}$ receptor (FP), and the oxytocin receptor (OTR). There is speculation that the regulation of these genes involves a common pathway, however this has yet to be determined. Most of the research into the regulation of the CAP genes has involved the role of estrogen (E_2) and progesterone (P_4), as there is a major hormonal shift in the uterine environment in preparation for labour. However, uterine activation has also been shown to occur via mechanical and immune factors, which are discussed below. Clearly, the onset of labour is a complex process that involves the delicate balance of many systems.

1.2.2 Endocrine system and parturition

In sheep, the signal for parturition is believed to arise from the maturation of the fetal hypothalamic-pituitary-adrenal (HPA) axis, which occurs during the last month of the 147-day ovine pregnancy.[10,11] Briefly, in response to fetal CRH, the fetal pituitary releases adrenocorticotropin hormone (ACTH). Subsequently, fetal cortisol is secreted from the adrenal, stimulating the placental enzyme cytochrome $P450_{c17}$ enzymes (17α hydroxylase and 17-20 lyase) expression. These enzymes convert pregnenolone (or C-21 steroids), to estrogens (or C-18 steroids), shifting the hormonal balance in the uterine environment.[13] This "withdrawal" of P_4 and increase in E_2 are thought to promote uterine activation and delivery through the synthesis of uterotonins and CAP genes, such as OT, OTR, Cx-43, and PGHS2.[14-16] Though this model is well-accepted in the sheep, evidence does not support the role of glucocorticoids as the primary stimulus for parturition in

rodents such as mice and guinea pigs, nor in humans.[16,17] However, there is some support for the involvement of the HPA system in the onset of human parturition, for CRH levels in the human have been strongly correlated to the timing of parturition.[18,19] The mechanism of CRH activity is not certain, though Smith et al have demonstrated in vitro that CRH stimulates the fetal adrenal to produce dehydroepiandrosterone-sulphate (DHEA-S), the androgen precursor to E_2.[21] Evidently, the androgen conversion to E_2, through CRH, is an important part of the physiological process that precedes parturition.

The decline in P_4 concentration has been stated as "the single most common endocrine event associated with parturition across species."[22] Though it may not specifically be a "decline" that occurs in every species, there is a "functional withdrawal" of P_4 action. In the human, where maternal plasma P_4 levels do not decrease, the P_4 receptor antagonist RU486 still disrupts labour and leads to premature delivery.[23] This suggests one of two things: either that relevant hormonal changes are occurring in a local paracrine fashion or, that the P_4 receptor may be of more physiologic relevance than circulating P_4 levels. Indeed, current research indicates that the profile of P_4 receptor isoforms changes at term to favor the expression of the PR-A, a dominant negative repressor of PR-B[24, 25]. The functional mechanism of P_4 and its receptor in the process of parturition is still under investigation.

It has been suggested that the balance, or ratio, of E_2 to P_4 is more important than actual hormone levels in regulating parturition.[26] Indeed, E_2 is responsible for many aspects of uterine activation. It is involved in promoting the synthesis of oxytocin receptors, alpha-adrenergic receptors (responsible for modulating calcium channels), prostaglandin $F_{2\alpha}$, prostaglandin E2, and Cx-43.[27] Interestingly, despite its many roles in the parturition process, E_2 is not mandatory for *parturition* to occur, but activation must take place for the *labour* process to proceed. In the rat model, an E_2 antagonist simply delays labour,[28] and women lacking placental aromatase (which is responsible for the conversion of DHEA-S to E_2), still deliver at term.[29] Evidently, the steroid hormones are important regulators of parturition, but their exact timing and mechanisms of action are still under investigation.

1.2.3 Mechanical stretch and parturition

The role of mechanical stretch in uterine activation and the onset of labour has received much attention. The increase in intrauterine volume and uterine stretch is thought to work to induce expression of the CAP genes and accelerate labour onset. Indeed, twin pregnancies, where the intrauterine volume (and consequently tensile stress) is high, have a significantly higher rate of preterm delivery than singleton babies.[30] Women with polyhydramnios also deliver their babies early compared to normal pregnant women.[31] Experimentally, this is supported by Manabe et al, who were successful in inducing labour within 24 hours in patients whose uteruses had been distended with a saline-filled balloon.[32]

Stretch is a major contributor to increased CAP expression and subsequent myometrial contractions. Specifically, Cx-43, OTR, and PGHS2 have all demonstrated responsiveness to stretch.[31,32] In rats that carried either a pup and placenta, or a tube (in a nongravid horn), mRNA levels of OTR and of Cx-43 increased at term.[32,33] However, the effect of this stretch mechanism appears to work in tandem with the endocrine environment, for the stretch-mediated increase in these CAP genes could be blocked by P_4. This demonstrates that there is a necessary interplay among the endocrine and mechanical factors at play in the uterine environment.

1.2.4 The immune system and parturition

The role of the immune system in the process of parturition, both term and preterm, is of unique interest because inflammatory cytokines are implicated in the progression of labour in both physiological (term) and pathophysiologic (preterm) conditions. Specifically, interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α are the cytokines most often associated with labour and delivery.[36] All three are found in amniotic fluid, amnion, chorion, decidua and myometrium, and are higher in tissues after labour than before labour onset.[35,36] They are also increased in times of intrauterine

infection. Interestingly, labour can be induced in rabbits[39] and rhesus monkeys[40] by exposure to various cytokines - an effect which can be prevented by certain anti-inflammatory cytokines. Whether this pharmacological intervention mimics the physiological cascade that occurs during normal labour is unknown. However, it is largely accepted that an inflammatory response is one of many systems that contribute to uterine activation.

In the labour process, cytokines have largely been associated with the induction of prostaglandins[41-44] and matrix metalloproteases (MMPs),[45] for both myometrial and cervical preparation for labour. However, their role in OTR induction has also recently been investigated, with conflicting results. Various transcription factors are stimulated in response to inflammatory cytokines, and several of these have response elements in the promoter region of the OTR gene (discussed below). It follows that such investigation into the molecular regulation of the gene should occur. Schmid et al[46] found that IL-1β and IL-6 negatively regulate OTR transcription in vitro, while Rauk et al found the opposite effect for IL-1β,[47] and Helmer et al found the opposite effect for IL-6. Further, Fang et al found that in uterine explants from pregnant rats (but not from non-pregnant rats) IL-6 treatment increased OTR mRNA.[48] Considering the variability in the data to date suggests a multifactorial system of gene regulation. The immune system is in delicate balance and its effects are probably tissue- and time-dependent. The immune system does play a role in CAP gene induction but its exact mechanism has yet to be determined.

1.3 OXYTOCIN

1.3.1 Overview

The hormone, oxytocin (OT) (its name derived from the Greek for "quick birth") is a potent uterotonin. It was first described in 1906 when Dale discovered the uterine stimulatory effect of a substance, later identified as OT, in the posterior pituitary gland.[49] Its role in lactation was also described by Ott and Scott, after injecting lactating goats

with this "pituitary extract" and measuring the milk secretion rate.[50] The nonapeptide structure of OT was later determined, and the Nobel Prize was awarded to Vincent Du Vigneaud for its synthesis in 1955.[51]

The human OT gene was cloned in 1983[52] and is found on chromosome 20p13. It contains three exons and 2 introns (Figure 2) and is located upstream of the vasopressin gene, with which it shares considerable homology. OT mRNA is transcribed first into a prohormone form, connected to its carrier protein, neurophysin I, via a Gly-Lys-Arg linker. Neurophysin I is cleaved from the prohormone by an endopeptidase, and the remaining polypeptide is further converted to the active hormone by carboxypeptidase B and α-amidating enzyme.[53]

OT is primarily produced in the supraoptic and paraventricular nuclei of the hypothalamus and stored in the posterior pituitary after migrating by axonal transport through the neurohypophyseal stalk. Upon secretion, OT enters the general circulation where, upon reaching its target receptor, signals one of various physiological responses. OT is well known to cause contraction of the myometrium[54] and breast myoepithelial cells[55], but is also associated with heightening sexual behaviour in both sexes,[54,55] promoting affiliation,[58] inducing maternal behaviour,[59] and causing cardiac relaxation.[58,59] Blood concentration of OT also increases in times of stress and high plasma osmolality, though its functions during these times are not fully understood.[60,61] The focus of this thesis is the OT/OTR system in the human uterine myometrium.

1.3.2 *Role of oxytocin in parturition*

The OT system is well-known to be an active part of the parturition process. The levels of both OT and its receptor increase dramatically at term in all models studied.[64-67] Pituitary release and maternal plasma concentration of OT do not increase significantly until parturition has already begun in both the rat[68] and in the human.[62,67] Maternal plasma concentrations of OT do rise somewhat nearing term and its pulsatile release is detectible if the frequency of samples is high enough. The significance of this pulsatile

release is not clear, though these spurts, combined with an increased nocturnal release, have been associated with increased myometrial contractility in the rhesus monkey.[70]

Reflexive secretion of OT from the pituitary, termed the Fergusson reflex, occurs when neuronal pathways are excited by uterine contractions.[71] However, the exact nature of the role of OT remains controversial. Even though OT is the strongest uterotonin known and is used to induce labour,[72] parturition may occur without OT. Women with a dysfunction of the posterior pituitary deliver normally[73] and mice devoid of the OT gene still deliver normally at term.[72,73] Interestingly, these mice are not able to lactate, indicating that OT itself is an essential requirement for lactation, while there may be an alternative or compensatory mechanism at play in the process of parturition. Combined with the fact that the half-life of OT in the blood is approximately 1.5 minutes,[76] this suggests one of two things. Either OT plays a smaller role in parturition than previously thought, or there is an alternate mechanism of OT production that contributes to the onset and progression of labour. Indeed, local production of OT has been demonstrated in many tissues of animal models and humans, including: human fetal membranes,[77] corpus luteum,[78] and rat uterine epithelium.[77,78] In rat uterine epithelium, OT mRNA has been found to increase over 150-fold at term over basal levels,[80] and this is 70-fold higher than hypothalamic OT mRNA.[79] And, human chorio-decidual OT mRNA expression increases 3 to 4 times around the onset of labour.[81,82] Together, these studies support a paracrine role for OT in the reproductive tissues suggesting that as a part of a paracrine network, OT mediates quick, neurologically independent actions approaching and during parturition.[83]

1.3.3 Regulation of oxytocin

The regulation of OT transcription appears to be largely under the control of E_2. In human chorio-decidual explants, OT mRNA levels increase after treatment with E_2,[16] while the in the overiectomized rat exposed to exogenous E_2, the uterine epithelium increased expression of OT mRNA.[84] Moreover, OT mRNA levels decline in the pregnant rat uterus after exposure to an E_2 receptor antagonist.[14] E_2 action may be

mediated by an imperfect palindrome hormone response element which is highly conserved among species. It is found approximately 160 bp upstream of the transcription start site of the OT gene and is a site potentially used by other regulators as well, such as thyroid hormone,[85] retinoic acid,[86] and the transcription factors chicken ovalbumin upstream promoter transcription factor COUP-TFI and steroidogenic factor-I (SF-1).

The role of P_4 as a regulator of OT gene expression is controversial. Lefebvre et al[84] demonstrated that it acts in a synergistic manner with E_2 to induce significant OT mRNA expression in the rat. When administered alone, it had no effect on OT. Likewise, administration of a P_4 receptor antagonist RU486 results in a decrease in rat uterine OT mRNA in late gestation.[28] However, these are counter-intuitive findings considering the decline in P_4 (or the increase in the E/P ratio) at term. The promoter region of the OT gene does not contain a P_4 response element, suggesting that the effect of this hormone is not mediated in direct genomic fashion, but may involve the participation of other transcription factors or higher order complexes. Perhaps a more pertinent role for P_4 in the OT/OTR system is its action on the receptor. This is discussed below.

Despite the increase in OT levels in the reproductive system at term, the increase in OTR levels may be even more prominent, providing the uterine tissue with a heightened sensitivity to OT. This suggests that the receptor for OT plays large role in sensitising the uterus to the parturition process. Evidently, the OT/OTR system plays an important role in the labour process.

1.4 THE OXYTOCIN RECEPTOR

1.4.1 Overview

The OTR was first described in the rat mammary gland in 1973 and soon after, was found in the human and rat uterus in the 1974.[87-89] The OTR 17 kb gene locus is a single copy mapped on chromosome 3 (3p25 - 3p26.2).[90] Interestingly, different sized OTR

mRNA transcripts are found in the breast (3.6 kb) versus the ovary, endometrium, and myometrium (4.4 kb), a feature which may prove to be important in its regulation. In fact, it has even been suggested that there are receptor isoforms of OTR but evidence confirming this is lacking.[91,92] The human OTR gene was cloned in 1992[93] and was found to contains four exons and three introns (Figure 3). It is a 43 kDa, 389 amino acid polypeptide that belongs to class I (rhodopsin-type) of the G-protein-coupled receptor (GPCR) family, largely characterized by seven alpha-helical transmembrane domains and an extracellular N-terminus and an intracellular C-terminus.[94]

The OTR is predominantly expressed throughout the tissues of the female reproductive system, namely the myoepithelial cells of the breast, myometrium, endometrium, amnion, chorion, decidua, ovary, and corpus luteum.[95] However, it is also found in the male reproductive system,[96] the kidneys,[97] the heart,[98] the vascular system[99] bone tissue,[100] and in areas of the brain.[101] As mentioned above, the primary known functions of the OT/OTR system involve milk-let down in the mammary glands and uterine contractions during and after labour. However, the presence of OTR in other tissues indicates it may play additional roles in the body.

Myometrial concentration of OTR increases dramatically at term, thereby increasing responsiveness of the uterus to OT. This dramatic increase has been shown in all models studied and in the chorion, amnion, decidua and myometrium. Numerous studies dictate that dramatic OTR mRNA and protein induction occurs, but an agreement on the exact amount of the increase has not been reached. Estimates range from a 27-fold increase in mRNA,[102] to a 180-fold increase in OT-binding.[103] There is even one estimate that states a 300-fold increase in mRNA.[104] These studies all used various models and experimental protocols to study the OTR increase which may account for the differences. However, it is widely accepted that the induction of OTR is dramatic and common to all species studied.

Interestingly, a common finding is that the most dramatic increase in OTR is not seen until labour has already begun, suggesting that the OT/OTR system may be more involved in the progression of labour than its initiation. However, Hinko et al found that

the increase in OTR mRNA in rabbit *amnion* preceded "by several days" the increase found in the decidua and myometrium.[105] Clearly, it is worth examining further the temporal and spatial regulation of the OT/OTR system in the reproductive tissues.

1.4.2 Mechanism of action of the oxytocin receptor

The structure of oxytocin lends itself to high affinity binding to the oxytocin receptor. Its nonapeptide structure is looped to create a disulfide bridge between the cysteine residues 1 and 6 (Figure 4a). This results in a looped N-terminus, which associates with the third extracellular loop of OTR, and a C-terminal tail which recognizes the first two extracellular domains.[106] On the extracellular surface of the OTR, there is an agonist-binding hydrophobic pocket created by the disulfide bridge between the first and second extracellular loops and by a cleft created by the "ring" of transmembrane domains. Ligand binding is facilitated by both specific residue association and hydrogen bonding with the disulfide bridge region. A schematic of the proposed ligand association of oxytocin and its receptor, adapted from Barberis et al, is shown in Figure 4b.[107] Gln residues in transmembrane (TM) domains II, III, IV, VI and a Lys residue in TM domain III contribute to ligand affinity.[108] Mutagenesis experiments done on the closely related vasopressin system can be extrapolated to the OT system, and indicate that OT Leu-8 is important for ligand specificity and associates with OTR residue F103. When rat vasopressin receptor Y115 (corresponding to the OTR residue F103) is mutated to F, there is a 19-fold increase in OT binding, switching agonist selectivity from vasopressin to OT.[109] Interestingly however, it is not the linear portion of the nonapeptide that contributes most to agonist selectivity, but rather the cyclic portion - of which Ile-3 is specific to OT. The second extracellular loop of OTR interacts with this cyclic region and residues Y209 and F284 are especially important.[110] Other residues important in OT binding can be seen in Figure 5.[95] The knowledge of OT binding activity has been used to develop both agonists and antagonists (such as Atosiban), with moderate success, in an attempt to manipulate the OT/OTR system.[111]

Following ligand binding, subsequent receptor activation (or conformational change to its active form) is facilitated through the exposure of a "polar pocket" by the shift of conserved D136 and R137 residues to expose buried sequences from the second and third intracellular loops to the cytoplasm.[112] G-protein recognition follows, carrying the OT signal into the cell in the form of an intracellular signalling cascade (Figure 6). The GTP-binding protein is a heterotrimer composed of $G\alpha_{q/11}$, Gβ and Gγ. When associated with the active form of the OTR, GDP is exchanged for GTP on the Gα protein, leading to its dissociation from the Gβγ complex.[113] Gα stimulates phospholipase Cβ (PLCβ) to hydrolyse phosphatidylinositol-4,5-bisphosphate (PIP_2) from the lipid bilayer to produce diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). The latter is responsible for the cytosolic influx of calcium (Ca^{2+}) stores from the sarcoplasmic reticulum (SR), while the former is an activator of PKC.

1.4.2.1 Activation of myosin light chain kinase (MLCK)

The rise in myometrial cytosolic calcium leads to muscle contraction. This occurs not only through IP_3-stimulated SR Ca^{2+} release, but also through receptor operated channels and voltage dependent L-type calcium channels on the cell membrane.[71] The additive increase in intracellular Ca^{2+} concentration causes smooth muscle contraction. This Ca^{2+}-dependent myocyte contraction occurs through activation of myosin light chain kinase (MLCK) (Figure 6).[114] For this to occur, Ca^{2+} binds to calmodulin, creating a complex which associates with the catalytic subunit of MLCK. MLCK then phosphorylates the regulatory myosin light chain (MLC_{20}), resulting in actin binding and the activation of myosin ATPase. The presence of ATP and its hydrolysis results in shortening of the myofilaments and subsequent smooth muscle contraction.[71, 115]

When Ca^{2+} levels fall, MLCK is deactivated and subsequent relaxation occurs. The contractile machinery is also under regulation by a balance of kinases and phosphatases. Relaxation occurs through phosphorylation of MLCK by calcium-calmodulin-dependent protein kinase II or cyclic AMP and GMP-dependent protein kinases.[71] Further, dephosphorylation of MLC is primarily regulated by myosin light chain phosphatase

which dephosphorylates myosin.[116] The intricacies of smooth muscle contraction and the balance between the proteins involved is complex and warrants further research.

1.4.2.2 Activation of protein kinase C (PKC)

As mentioned above, PKC is stimulated by the release of DAG from PIP_2 hydrolysis. PKC is an ubiquitous kinase involved in many cellular mechanisms, including differentiation, proliferation, secretion, gene expression, and smooth muscle contraction.[117] Many of these effects are isozyme- or cell-type-specific. Of interest in the myometrium is both its role in contraction and as a mediator of gene expression (Figure 7). It contributes to contraction via activation of voltage-gated L type Ca^{2+} channels[118] and by the phosphorylation of various contractile proteins like caldesmon and calponin.[119, 120] A study by Morrison et al showed with the use of PKC inhibitors that PKC activation was required for OT to produce maximal contraction in human myometrium.[121] However, other investigators have found the opposite to be true. Phillippe et al studied the role of PKC in rat uterine contractions and found that stimulation of PKC using the phorbol ester TPA suppressed OT-induced contraction.[122] Clearly, the role of PKC as a mediator of myometrial contractions is complex and may involve a temporal pattern of regulation. Further studies are needed to address this issue.

The role of PKC as a mediator of signal transduction pathways leading to gene expression presents the likelihood of alternative OT-mediated effects. A major signalling pathway stimulated by PKC is the mitogen activating protein kinase (MAPK) pathway. It modulates the expression of various genes through transcription factor regulation. The MAPK signalling pathway is perhaps best known for its role in regulating genes involved in growth and differentiation. However, it also plays a role in regulating genes involved in the birth process. Indeed, Ohmichi et al showed that OT stimulated MAPK activity in myometrial cells.[123]

The active form of PKC stimulates c-Raf which targets the MAPK cascade, leading finally to the activation of phosphorylation-sensitive transcription factors, such as

Activator Protein- 1 (AP-1). In addition, the Gβγ subunit of the G-protein trimer, also stimulates the MAPK kinase pathway. Interestingly, there is potential for a convergence of the MAPK signaling pathways originating from "traditional" AP-1 stimulators, which mainly work through tyrosine kinase receptors, and the OT/OTR system (Figure 7). AP-1 is discussed below in more detail.

1.4.3 Genomic regulation

As discussed above, the dramatic upregulation of OTR at term is mediated by several systems, namely endocrine, mechanical, and immunological. It is thought that their effects on gene transcription are mediated primarily by the balance of regulatory transcription factors.

The OTR gene contains a large 5'-flanking non-coding, or promoter region, which contains several potential binding sites for regulatory transcription factors (Figure 3). This suggests a complex mechanism of transcriptional regulation. There are many binding sites on the OTR promotor and include: one Sp1 site, three nuclear factor interleukin-6 (NF-IL6) sites, one nuclear factor kappa B (NF-kB) site, two signal transduction and activator of transcription-3 (STAT-3) sites, one Ets site, one half cAMP response element (CRE/2), and two activator protein-1 (AP-1) sites.[90, 124] Also, even though the human OTR promoter does not contain a consensus E_2 response element (ERE), there are two 5'- and one 3'-half E_2 response elements (EREs).

Studies regarding direct transcription factor regulation of OTR are still in early stages. However, current research has determined areas of the OTR promoter region that are highly responsive. For example, Hoare et al, found that an area in the proximal promoter region between -85 and -65 was highly responsive to the Ets factor GABPα/β when co-transfected with the AP-1 dimer.[124] This group had previously shown that in the same cell line, OTR was upregulated by serum and this effect could be prevented by pre-treatment with a PKC inhibitor.[125] These experiments were done in the breast cell line Hs578T and the data may or may not be extrapolated to OTR regulation in the uterine

tissue. However, they certainly indicate the potential for dramatic increases in transcription in response to the PKC pathways.

As discussed above, the inflammatory cytokines are associated with both term and preterm labour. There have been limited studies of cytokine regulation of OTR, with varying results. However, there is support for the involvement of some transcription factors. Indeed, Schmidt et al found that the regulation of OTR was dependent on the -1203/-722 region of its promoter, and may involve NF-IL6,[46], while Rauk showed evidence of STAT-3 involvement.[126] In addition, it has been demonstrated that TNFα activates AP-1 through the recruitment of members of the MAPK family, JNK and p38.[127] A common endpoint mediator of the CAP gene induction, perhaps through these cytokines, is an appealing hypothesis. However, as it stands, OTR regulation involves a complex tissue- and time- specific response of regulatory transcription factors. A more detailed analysis of the cytokine signal transduction in uterine myocytes is required.

Upregulation of OTR in response to glucocorticoids has been demonstrated in the rabbit model.[128] OTR mRNA increased significantly in pregnant rabbits injected with glucocorticoids. This also resulted in preterm labour.[129] In addition, Hinko et al demonstrated in rabbit amnion cells in culture, that this increase in OTR mRNA by glucocorticoids was potentiated by treatment with forskolin (a stimulator of adenylate cyclase).[128] The same group later studied the molecular basis of this effect and concluded that the primary source of the OTR increase was due to transcriptional, as opposed to translational or post-translational processing.[130] Indeed, mutation of a CRE has previously been shown to eliminate the effects of the forskolin/cortisol induction, by preventing binding of the cAMP and the associated glucocorticoid receptor (GR).[131]

The steroid hormone E_2 is a strong regulator of OTR gene expression.[102] Both Murata et al and Fang et al have shown that the increase in OTR mRNA in the rat prior to parturition can be prevented with injections of the anti-E_2 tamoxifen.[28, 132] This E_2-dependence has also been determined in human primary cultures.[133] However, the exact genetic mechanism by which the effects of E_2 are mediated is not clear. As mentioned, there is no full ERE in the promoter region of the human OTR gene. Yet, there are three

½ ERE's which may confer E_2 responsiveness through a mechanism similar to that found in the chicken ovalbumin gene, which also contains only ½ EREs.[134] Alternatively, the effects of E_2 may be mediated through transcription factors other than E_2 receptor (ER) or through cofactors.[135] E_2 increases mRNA expression of the transcription factors cJun and cFos,[136, 137] members of the AP-1 family, with which the ER can form a complex.[138, 139] The ER can also associate with the transcription factor Sp1 and this complex has been previously found to induce heat shock protein 27 gene expression.[140] It remains to be determined whether these higher order complexes play a role in OTR transcription.

P_4 is also a regulator of the OTR system but involves a different mechanism. Larcher et al found that P4 administration in the rat model indeed significantly decreased OT-binding sites but did not affect the E_2-induced rise in OTR mRNA.[102] This suggests that the effects of P_4 are mainly at the post-translational level. This is discussed in more detail below. In contrast however, Fang et al observed that administration of the P_4 receptor antagonist RU486 increased OTR mRNA expression (and even led to preterm delivery), supporting an inhibitory role for P_4 on OTR transcription.[28]

Another important aspect of regulation at the transcriptional level is the varying methylation state of the OTR gene.[141] Methylation of the cytosine residue (5'-CpG-3') is associated with repressed transcription and in certain genes there are "CpG islands," or potential areas of high methylation which contribute to their regulation. Alternatively, hypomethylation may lead to increased gene expression. Presumably, access to the promotor region by transcription factors is decreased when methylation is high. Interesting studies by Kusui et al and Mizumoto et al found that methylation state of OTR in the liver (where OTR is perpetually inactive) was significantly higher than in OTR found in reproductive tissues, where OTR is inducible. Interestingly, they found that there was a slight decrease in OTR methylation in uterine samples from pregnant women versus non-pregnant.[142] Further studies are needed to explore this phenomenon.

1.4.4 Non-genomic regulation

At term, the massive increase in OTR levels is largely attributed to regulation at the transcriptional level.[143] However, there may also be significant post-translational regulation of the OT/OTR system. In fact the desensitization, internal trafficking, and attenuation of G-protein-coupled receptors (GPCR) by their ligands or other factors, all contribute to receptor, and ultimately cellular, responsiveness.[144]

It is well established that OT exposure promotes homologous desensitization of OTR.[145] This has been demonstrated in both cultured cells[146, 147] and in primary tissues obtained from women undergoing OT treatment.[148] Interestingly, this desensitization appears not to affect actual protein levels but rather receptor responsiveness, suggesting there is important regulation occurring on the membrane level.[146]

The exact mechanism by which desensitization occurs is not known but current hypotheses suggest that OT causes a conformational change in its receptor that, aside from carrying the signals into the cell required for muscle contraction, exposes intracellular domains containing potential phosphorylation sites.[149] (Figure 5) These sites may be bound and phosphorylated by various kinases, such as the G-protein receptor kinases (GRKs), PKC, and/or PKA. Sites for protein kinase G, casein kinase Iα and calmodulin kinase II have also been found.[149] It is thought that phosphorylation of the receptor causes a further conformational change leading to desensitization and/or internalization. In the case of GRKs at least, phosphorylation of a GPCR targets it for arrestin binding.[150, 151] Arrestins function to desensitise GPCRs by uncoupling them from their respective G-proteins and signals them for internalization.[152, 153] Interestingly, the activity of the GRKs is enhanced by PIP_2, Gβγ, and PKC.[154] Phaneuf et al showed that OTR responsiveness to OT is reduced by PKC stimulation, and it may be that this occurs via the GRKs.[155] However, it appears as though there is a cell type-specific response of the OT/OTR system to PKC stimulation. Strosser et al studied OTR binding activity in cultured rat hypothalamic neurons and astrocytes and found exact opposite response to PKC stimulation by TPA.[156] In rat neurons, OT binding was increased in response to TPA treatment, and in astrocytes it was decreased - the latter being in agreement with Phaneuf, above. Both effects were abolished if pre-treated with a PKC inhibitor. These

differences could be due to the involvement of different PKC isoforms. This highlights the importance of cell-specific context when studying this system.

The hormone P_4 has been demonstrated to regulate OTR in a genomic fashion (as discussed above), but also in a non-genomic manner. P_4 has been shown to associate directly with OTR at the membrane level to inhibit OT binding.[157] In ovariectomized rats where E_2 injections upregulate OTR mRNA and subsequent OT binding, cotreatment with P_4 decreased OT binding, but did not inhibit mRNA induction.[79,101] Other experiments show that P_4 attenuates the actions of GPCRs at concentrations in the micromolar range, concentrations that may be physiological in steroidogenic tissues such as the pregnant uterus.[95] It is necessary to keep in mind the endocrine context of myometrial cells, for the changes at term in P_4 levels may significantly affect OTR function. Of note, is that these non-genomic effects of P_4 are fast and reversible.

Another membrane-associated modulator of OTR function is cholesterol. Areas of the plasma membrane high in OTR are associated with high levels of cholesterol and a putative cholesterol-docking domain has been identified on the extracellular loops of OTR.[158] OTRs found in cholesterol-rich microenvironments are stabilized in their high-affinity state. Interestingly, P_4 interferes with cholesterol synthesis and trafficking, which suggests a possible mechanism for the maintenance of uterine quiescence throughout pregnancy.[158]

By examining the structure of OTR, it appears as though the N-glycosylation state of OTR may play an important role in its function and ligand selectivity. However, Kimura et al performed a set of experiments that showed that this is not the case. They developed HeLA cell lines expressing OTR in various N-glycosylation states and determined that their binding capacity, ligand affinity, or receptor trafficking were not affected.[159] Interestingly however, the molecular weight of OTR is greatly affected by its glycosylation state, varying from approximately 70 kDA (determined by immunoblot from uterine tissue) to the estimated molecular weight of 43 kDa. This may provide a clue as to the current challenges in OTR immunoblotting.[104]

1.5 ACTIVATOR PROTEIN-1

1.5.1 Myometrial AP-1

Activator Protein-1 (AP-1) is a dimerized transcription factor made up of members of the Fos and Jun proto-oncogene families. AP-1 binds to putative regulatory sites in various gene promoter regions. The AP-1 binding site is known as a TPA-response-element (TRE) and this is discussed in more detail below. Concentrations of the AP-1 proteins have previously been shown to increase at term in rat myometrium.[160] This increase has been shown to be mediated by both E_2,[159,160] and by stretch.[163] There are other mediators of AP-1 induction (such as cytokines and growth factors), but these have yet to be studied in the context of parturition.

The increase in myometrial AP-1 occurs prior to the increase in both OTR and Cx-43.[164, 165] AP-1 is a potential common mediator of CAP gene induction, for consensus TREs are found in the promoter regions of several CAP genes. In fact, it has been demonstrated that Cx-43 is highly responsive to TPA treatment and that this was dependent on a specific AP-1 binding site.[166] The role of AP-1 on Cx-43 regulation is well-established, but studies of AP-1 regulating human OTR transcription are few. Bale et al have demonstrated that the rat OTR gene is highly responsive to TPA treatment, and even more so when co-treated with forskolin.[167] The promoter region of the rat OTR gene is organized quite differently than that of the human and also contains three, rather than two, AP-1 binding sites. However, the same group did demonstrate in MCF7 human breast cancer cells that TPA treatment caused a forty-fold increase in binding of a radio-labelled oxytocin analogue, [^{125}I]-OTA (ornithine vasotocin) after 24 hours.[167] Futher, Hoare et al identified a GABP binding site in the OTR promoter of the human breast cell line Hs578T that induced transcription. The activity of this site was potentiated by AP-1.[124]

The involvement of AP-1 in the activation of OTR is a plausible and supported hypothesis. In fact, we suggest that there is a unique pathway by which the OT/OTR

system may drive its own regulation. As mentioned above, induction and activation of AP-1 primarily occurs via protein kinase cascades. These cascades are a potential point of convergence of the AP-1 and OTR signal transduction pathways. PKC is a by-product of OT signal transduction, and subsequent stimulation of AP-1 may lead to an increase in OTR, thereby increasing the available pool of OTR. In this manner, we propose that OTR lends itself to a "feed-forward" mechanism (Figure 8).

1.5.2 Overview of AP-1

As mentioned, AP-1 is a dimerized transcription factor made up of proteins from the Fos and Jun proto-oncogene families. In turn, Fos and Jun belong to the family of bZIP proteins, which is characterized by a basic leucine zipper region. This region serves to anchor the dimer together via its circular alpha-helix region where every seventh amino acid is a leucine. Typically, AP-1 is composed of either homodimers of the Jun (cJun, JunB, JunD) family or heterodimers composed of Jun with the members of the Fos family (cFos, FosB, Fra1, Fra2).[168] Other transcription factors may also associate with Jun and Fos, such as the activating transcription factor (ATF) and CRE binding proteins (CREB) bZIP proteins.[169, 170] Further, there has been evidence showing that the p65 monomer of nuclear factor-kappa B (NFkB) can trimerize with the AP-1 dimer, creating a unique complex of transcription factors with biologic activity.[171] Diversity among the AP-1 dimer associations contributes to differences in their binding, activation, and regulation of gene products - a complex issue which is a large area of research.[170, 172] The focus of this discussion is on the cJun and cFos proteins, the representative members of the Jun and Fos families.[173]

AP-1 was first discovered in 1987 as an essential transcription factor in regulating the expression of human metallothionein IIa.[174] The sequence of the AP-1 DNA binding site soon followed upon discovery of its responsiveness to the tumor promotor phorbol ester known as TPA (12-*O*-tetradecanoylphorbol-13-acetate).[175] The consensus sequence of this binding site is now known as a TRE (TPA-response-element), and it's coding region is: 5'-TGA$^{G}/_{C}$TCA-3'. It is important to note that heterodimers bind with greater affinity

than homodimers to the AP-1 DNA binding site. Further, binding of atypical dimers also occurs at other similar sequences such as at CREs (or cAMP response elements, 5'-TGACGTCA-3').[170]

The induction of *jun* and *fos* is rapid, consistent with the profile of early response or immediate-early genes (Figure 9). Upon activation, transcription is rapid and may be detected within 15 minutes, while post-translational modification of existing proteins also contributes to AP-1 induction. Activation can occur in response to growth factors, cytokines, UV irradiation, hormones, phorbol esters, heat shock, and cAMP.[168] Many of these stimuli act via protein kinase cascades, which act to stimulate Fos and Jun in different ways.

1.5.3 The regulation of cJun and cFos

At the transcriptional level, *cjun* is largely regulated by its own gene product, facilitating its own rapid upregulation upon stimulation (Figure 9). The *cjun* promoter region contains two TREs, which promotes a positive-feedback loop. cJun is also regulated by post-translational modification by phosphorylation, which contributes to its rapid induction. MAPK activation results in the phosphorylation of two important residues on the N-terminus of the cJun protein. These residues, serine 63 and 73, are located within the cJun transactivation domain. They are phosphorylated by the MAPK Jun N-terminal kinase (JNK) or p38 which are part of the MAPK pathways. This phosphorylation of cJun is required for transcriptional activation and is thought to facilitate the recruitment of CREB-binding protein (CBP), a potent coactivator involved in the recruitment of the basal transcriptional machinery.[176]

The C-terminus of cJun is also involved in its regulation. A similar MAPK cascade leads to the *de*-phosphorylation of a series of residues on the C-terminal end of the cJun protein, next to its DNA-binding domain. In nonactivated cells, threonines 231 and 239, and serines 243 and 249, are maintained in the phosphorylated state, inhibiting DNA-binding. Evidence supports that phosphorylation is maintained by Glycogen Synthase

Kinase (GSK)-3 and/or Casein Kinase II (CKII),[177] but the mechanism by which their down-regulation occurs upon stimulation is unclear. Interestingly, it has been proposed that phosphorylation of the cJun N-terminus transactivation domain renders the C-terminus end a poor substrate for kinases.[178] This indicates a cooperative mechanism among the branches of the PKC signalling cascades, resulting in cJun activation.

The regulation of cFos occurs primarily at the level of transcription (Figure 9). Upon stimulation, *cfos* induction is primarily induced by SRF (Serum Response Factor) which responds to PKC/MAPK signaling. SRF binds to the SRE sequence (Serum Response Element) in the *cfos* promoter and activates transcription. Interestingly, cFos differs from cJun in that it is *negatively* regulated by its own gene product.

Upon activation and dimerization, AP-1 will bind to its consensus sequence via its positively charged DNA binding domain, affecting transcription. Transcription factors may do this in several ways. They may contribute to loosening the chromatin structure to increase DNA affinity for the basal transcription machinery. Alternatively, they may induce a conformational change to alter accessibility (either positively or negatively) of the promotor to other transcription factors. Further, they may facilitate the formation of higher order complexes among transcription factors to bring the basal machinery in association with the transcription-start-site. In most genes, one or more of these processes is essential for transcription to occur. Because the promoter regions for many genes, including that for OTR, have multiple binding sites for many different transcription factors, gene regulation is highly complex. However, before understanding how AP-1 may regulate OTR we must first ask if it indeed plays a role. The experiments in this thesis have addressed this question.

1.6 HYPOTHESES

Activator Protein-1 regulates expression of the oxytocin receptor gene in human uterine myocytes in vitro.

The effects of AP-1 on OTR were examined in two ways.

1.6.1 Specific Aim One: TPA treatment and its effect on OTR

The **first aim** of the project was to determine the effects of AP-1 on OTR expression and function using phorbol ester (TPA) treatment to induce and activate AP-1. The effect of treatment on both AP-1 and OTR expression and function was examined.

1.6.2 Specific Aim Two: Adenoviral transfer of AP-1 proteins and its effect on OTR

To corroborate the results of aim one, the **second aim** of the project was to determine the effect of AP-1 on OTR expression and function by overexpressing AP-1 proteins using adenoviral infection technology. Results were examined as in Specific Aim One.

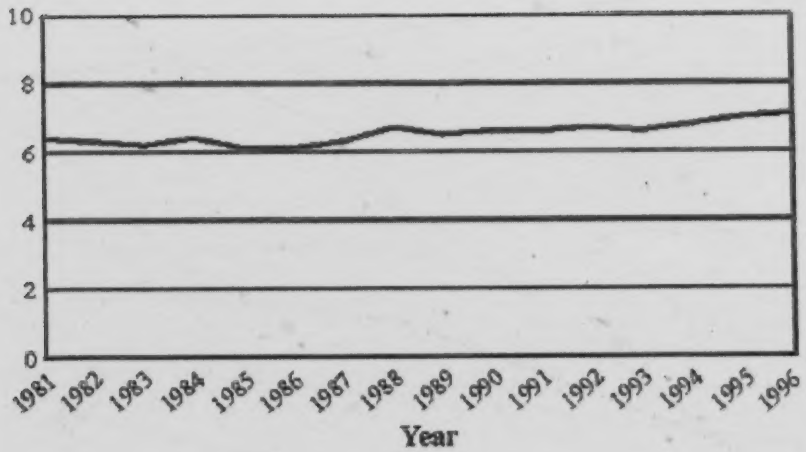


Figure 1. Preterm births per 100 live births in Canada, demonstrating the continued rise in preterm birth rate despite years of research. From Arbuckle et al.[3]

Cause	Frequency
Spontaneous preterm labour	30-50 %
Multiple pregnancy and associated complications	12-28 %
Preterm prelabour rupture of membranes (pPROM)	6-40 %
Hypertensive disorders of pregnancy	12 %
Intrauterine growth restriction	2-4 %
Antepartum haemorrhage	6-9 %
Miscellaneous - cervical incompetence, uterine malformation	8-9 %

Table 1. Causes of preterm delivery. From Slattery and Morrison.[2]

An example of how the causes of preterm birth can be categorized.

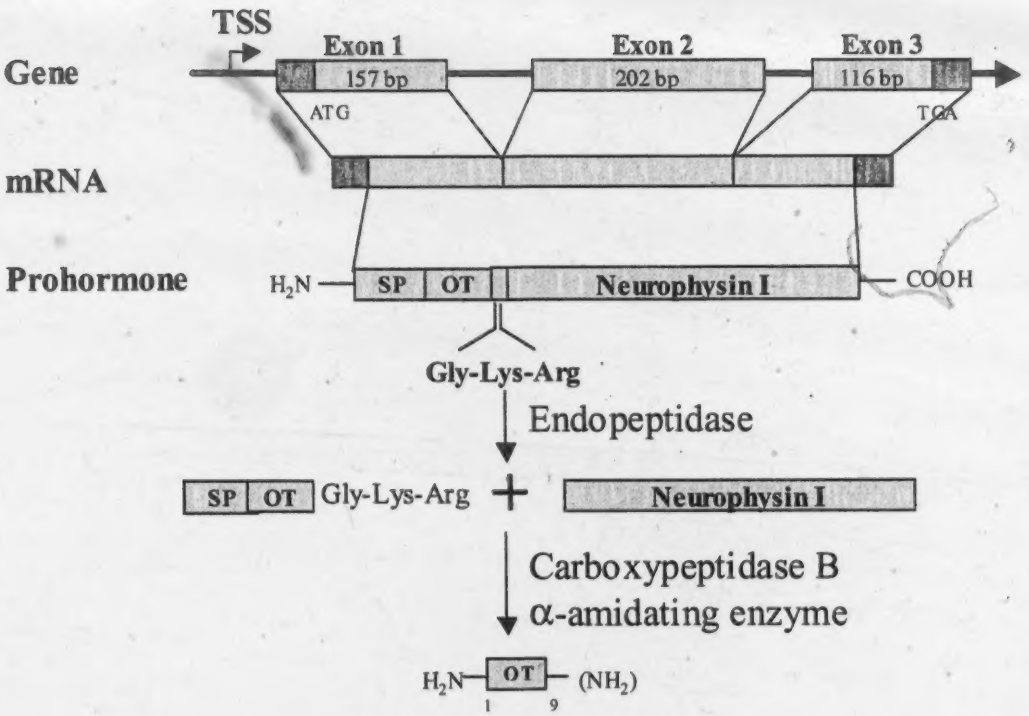


Figure 2. The oxytocin gene and its transcript. Adapted from Mitchell and Schmid,[179] Gimpl,[95] and Higuchi.[180]

Ex, exon; TSS, transcription start site; SP, signal peptide.

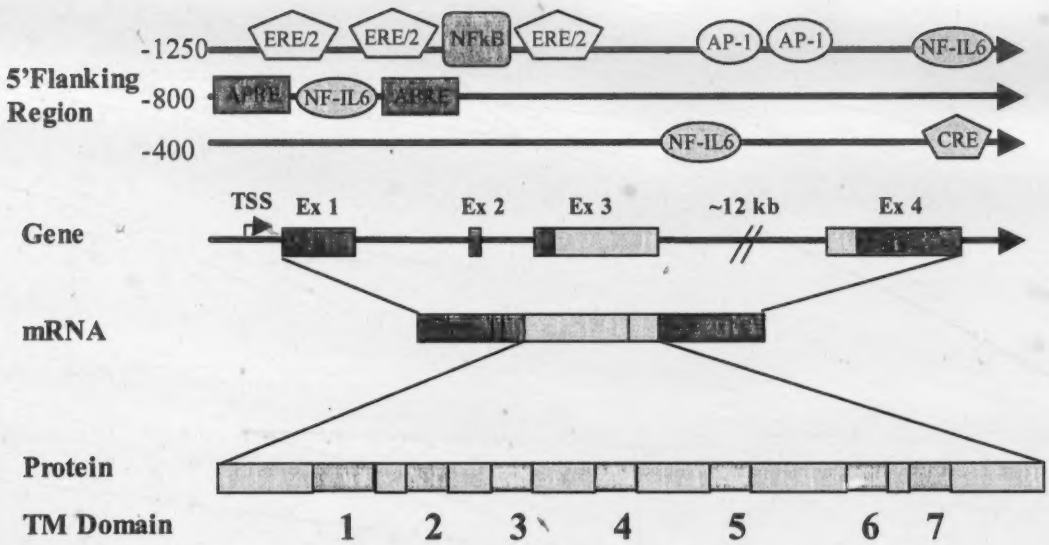
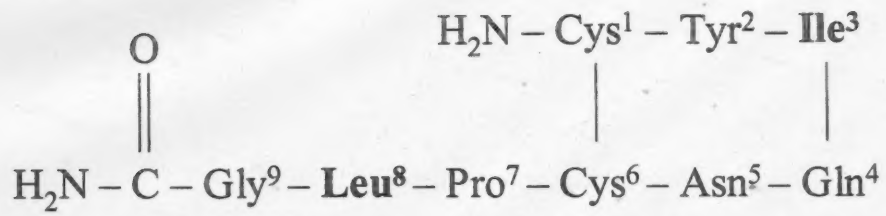


Figure 3. The oxytocin receptor and its transcript. Adapted from Mitchell et al[179] and Hoare et al.[124]

AP-1, Activator Protein 1; CRE, cAMP response element; NFkB, Nuclear Factor kappa B; APRE/STAT3; ERE, E_2 Response Element; NF-IL6, Nuclear factor- interleukin 6; TM, transmembrane domains; TSS, Transcription Start Site; Ex, exon.

A)



B)

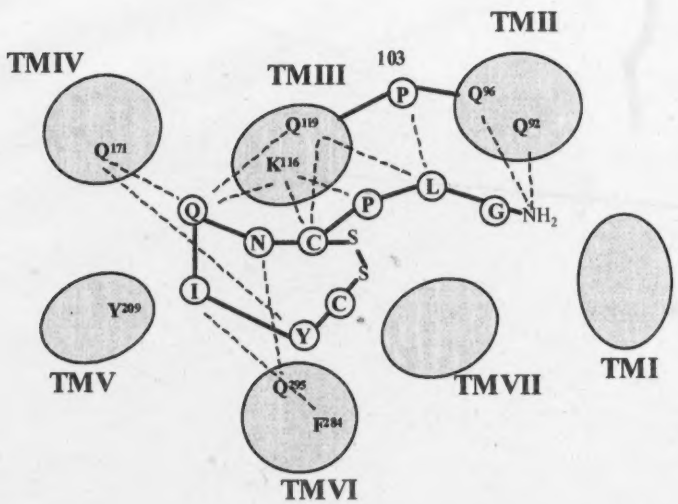


Figure 4. A) The amino acid structure of oxytocin. B) Proposed three-dimensional representation of oxytocin interacting with its receptor transmembrane domains.

In **A)**, residues differing from vasopressin are shown in bold. Adapted from Shojo et al.[181] **B)** Adapted from Barberis et al.[107]

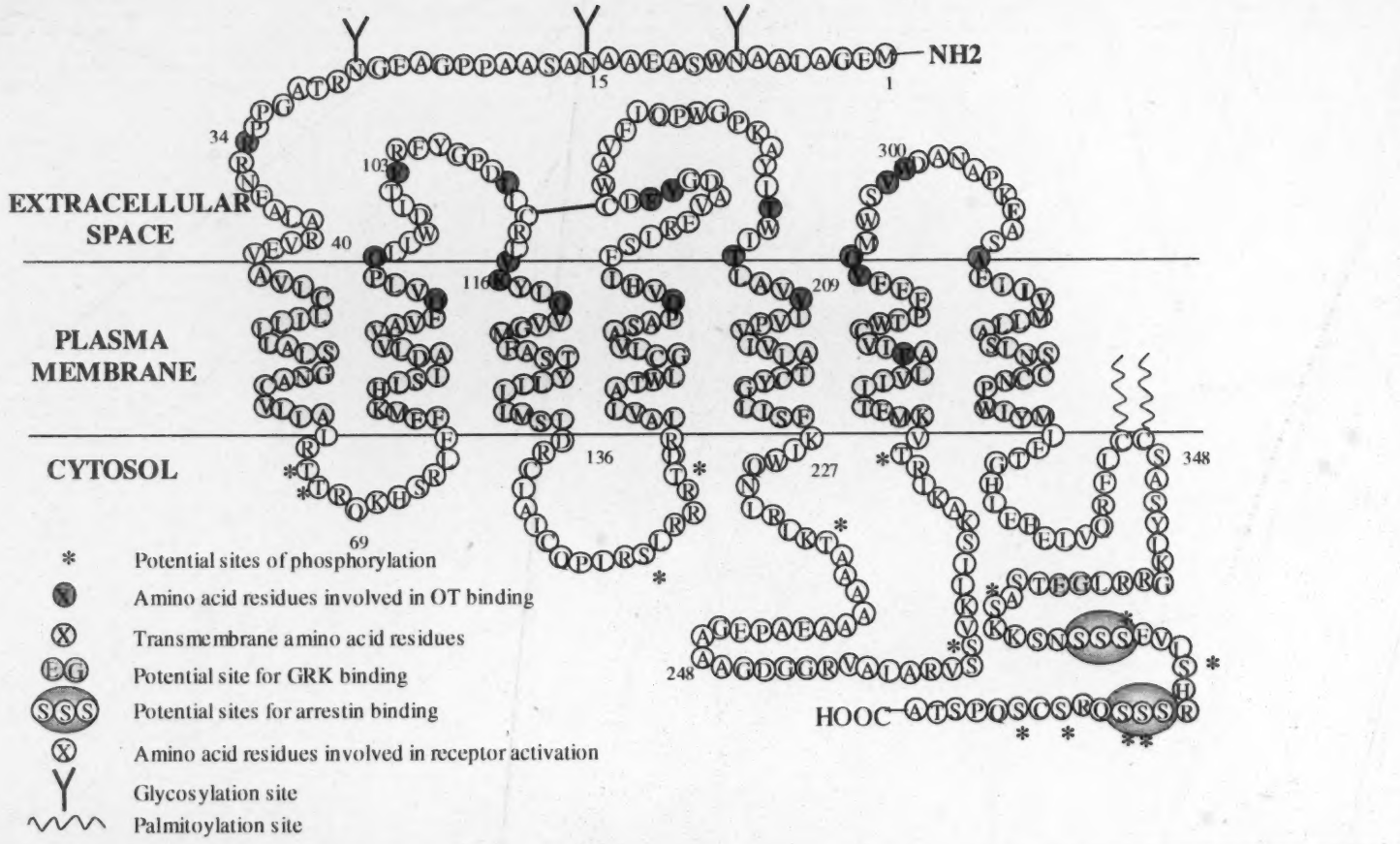


Figure 5. Human oxytocin receptor. Adapted from Gimpl et al,[95] and Plested et al.[149]

Schematic of the human oxytocin receptor, indicating amino acid residues involved in ligand binding, receptor activation, internalization, and phosphorylation.

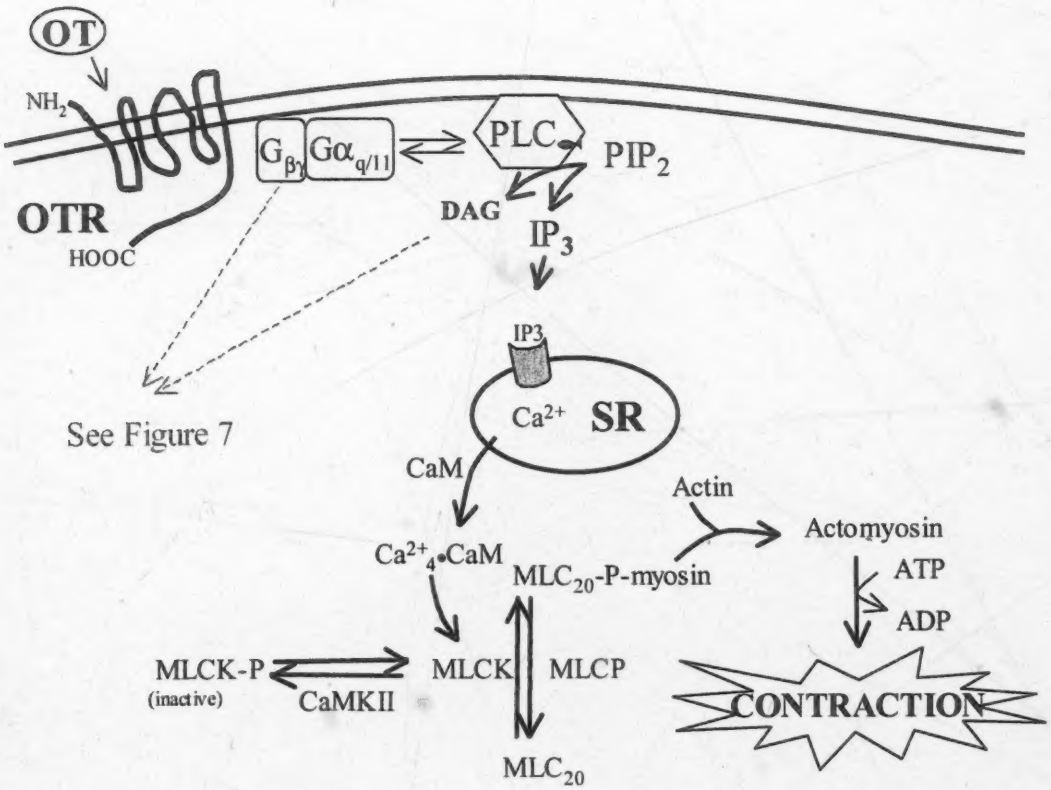


Figure 6. Oxytocin-mediated smooth muscle contraction.

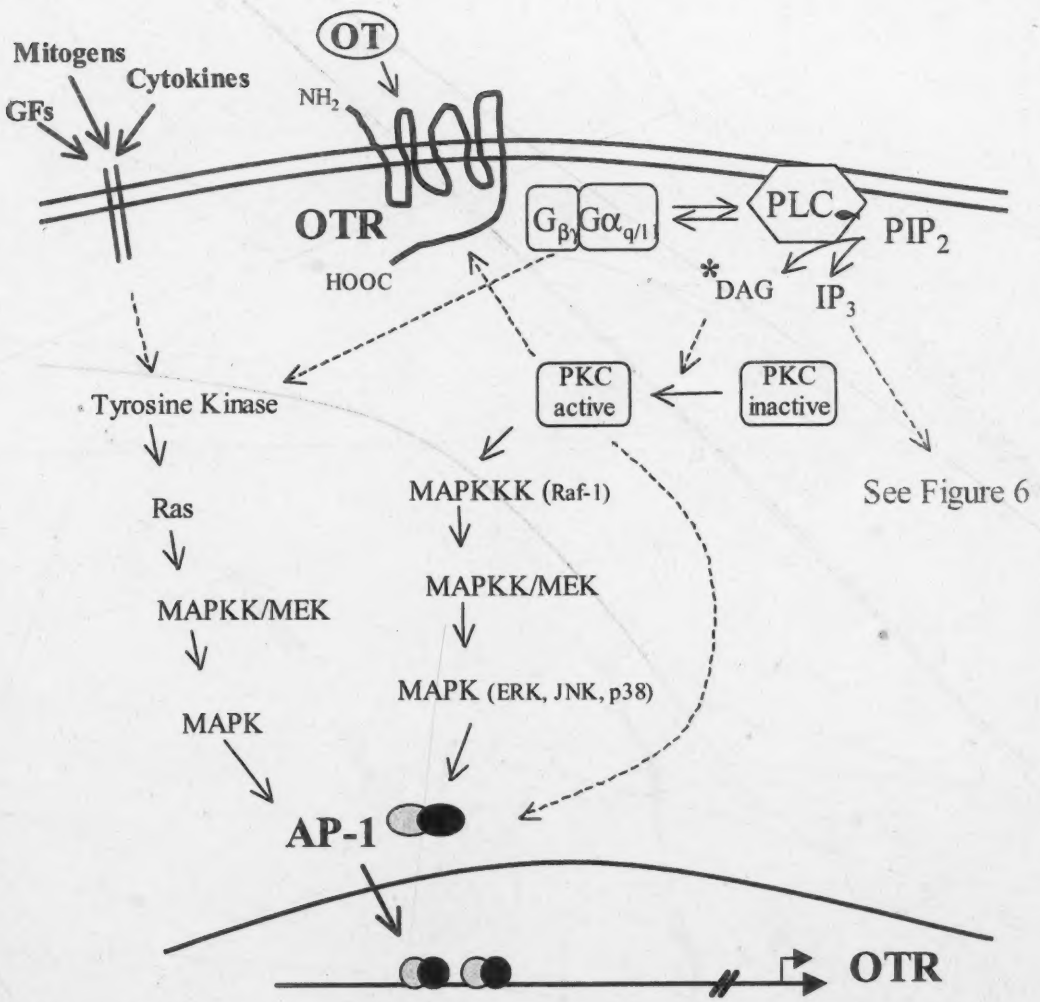


Figure 7. Potential convergence of oxytocin-mediated signaling in regulating AP-1.

* TPA acts as an analogue of DAG

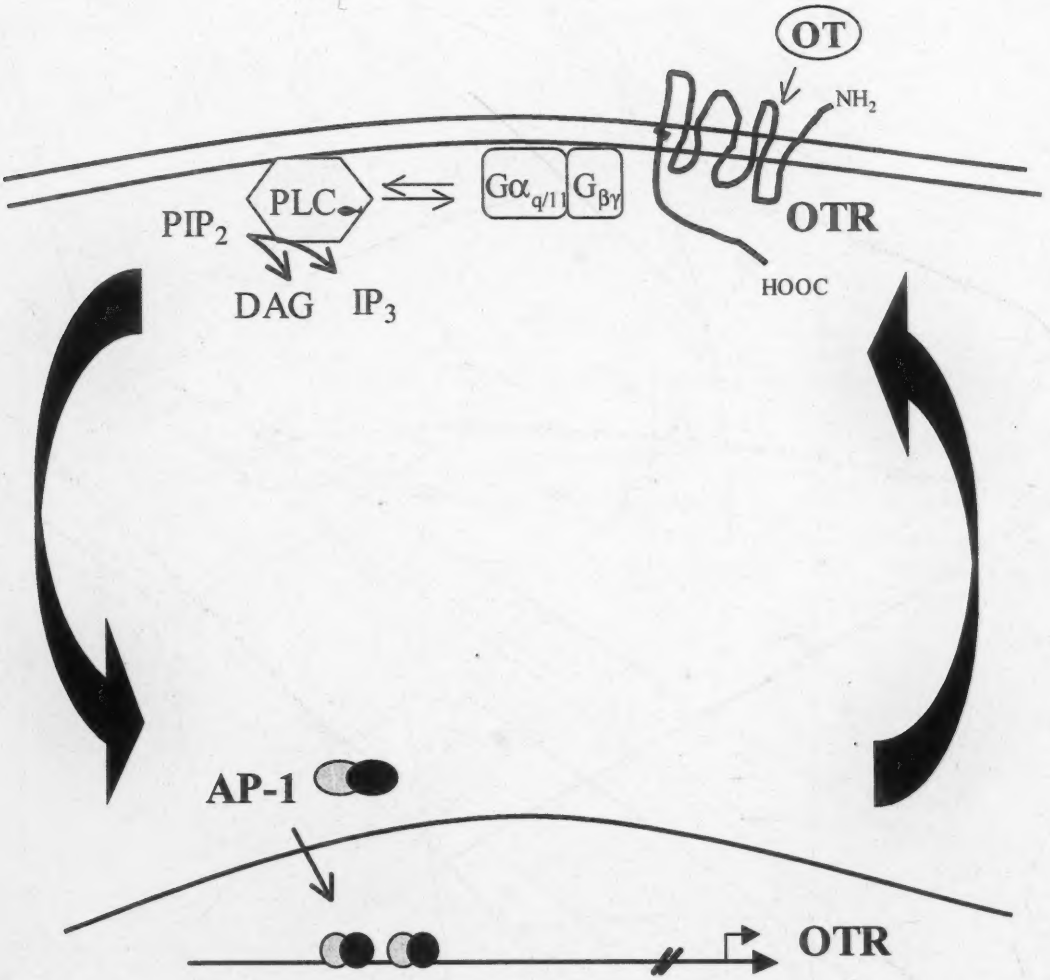


Figure 8. Potential "feed-forward" mechanism of oxytocin mediating the upregulation of its receptor.

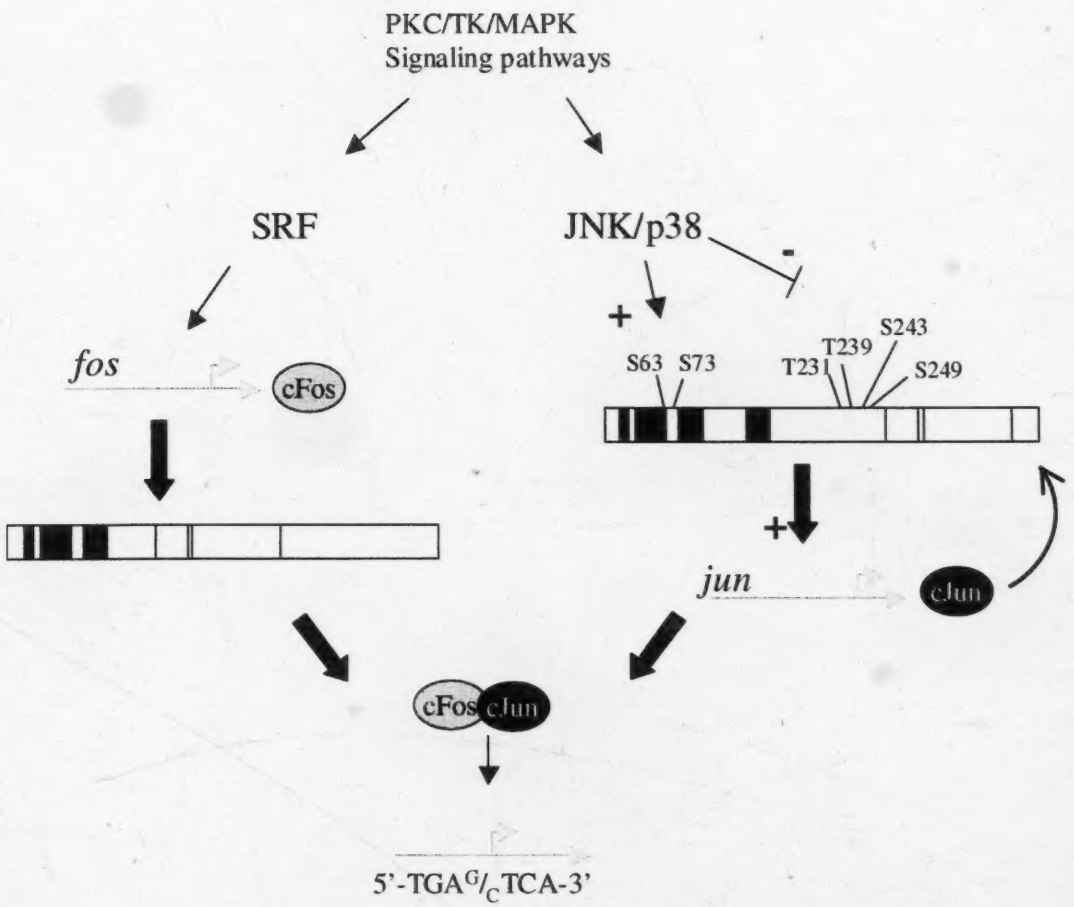


Figure 9. Induction and activation of the AP-1 proteins cJun and cFos.

■ Transmembrane Domains Basic Domains Leucine Zipper Domains

CHAPTER 2. EXPERIMENTAL DESIGN, METHODS, AND STATISTICS

To investigate the hypothesis, two approaches were used. A myometrial cell line, ULTR, was grown in culture and either treated with the phorbol ester TPA or was infected with the adenoviral vector for the AP-1 protein cJun (Figure 10).

2.1 AIM ONE: EXPERIMENTAL DESIGN

Cells were treated with TPA for 0.5 hours to stimulate PKC and assessed for changes in cJun expression (via immunofluorescence and western immunoblotting) and function (via an ELISA-based assay). In addition, cFos expression was evaluated by western immunoblotting. Subsequently, cells were treated with TPA over a 24 hour time course and RNA was extracted to measure changes in OTR mRNA by quantitative real-time RT-PCR. Further, binding assays were performed after TPA treatment to assess [^{3}H]-OT binding. Immunofluorescence was also performed for OTR and caveolin (used as a membrane marker) to observe any changes in OTR localization in response to TPA treatment.

2.2 AIM TWO : EXPERIMENTAL DESIGN

Cells were infected with the adenoviral vector AdcJun to overexpress cJun. First, the effect of AdcJun infection on changes in cJun expression was determined by western immunoblotting and immunofluorescence. Subsequently, the effect of AdcJun overexpression on OTR mRNA expression was examined by quantitative real-time RT-PCR.

2.3 MATERIALS AND METHODS

2.3.1 Cell line

Immortalized human myometrial cells (ULTR) were provided by Dr. J. K. McDougall's labouratory (Fred Hutchison Cancer Research Centre, Seattle, WA, USA).[182] These cells were obtained from a non-pregnant woman and immortalized with a recombinant retroviral construct containing the E6 and E7 open reading frames of human papillomavirus type 16. According to Perez-Reyes, they sustain long-term passage (up to 180 population doublings) with relatively little change in morphology.[182] All cells used in the present experiments were between passages 16 to 24. These cells have been characterized in the labouratory and are known to express actin (Figure 11), and receptors for E_2, P_4, FP, and oxytocin (BF Mitchell, unpublished data).

2.3.2 Cell culture

ULTR cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco), supplemented with 10% Fetal Bovine Serum (Invitrogen) and 1% Antimycotic (Invitrogen) at 37°C and 5% CO_2/95% Air. Cells were grown to approximately 80% confluence in either T75 flasks (Corning) for nuclear extractions, 6-well plates (Corning) for RNA extractions and total protein extractions, 12-well plates (Corning) for the binding assays, and 8-chamber slides (VWR) for immunofluorescence. TPA (Upstate Biotechnology) was diluted in DMSO and used to treat cells at a concentration of 100 ng/mL for up to 24 hours.

2.3.3 Nuclear extractions

After treatment, nuclear proteins were extracted according to the protocol in Schreiber et al.[183] Cells were washed 2-3 times in ice-cold PBS and scraped into 1.5 mL microfuge tubes, after which they were spun down at 1500 x g for 3 minutes at 4°C. The supernatant was removed and the pellet gently resuspended in 400 µl ice-cold Buffer A

(10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mL EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF), after which the cells were left to swell on ice for 15 minutes at 4°C. Twenty-five µL of Non-idet 10% NP-40 was added and the mixture vortexed vigorously for 10 seconds. The homogenate was centrifuged for 30 seconds at 14,000 g in a microfuge and the supernatant removed. The pellet was resuspended in approximately 50 µL (or half the 'pellet cell volume') of ice-cold Buffer C (20 mM HEPES, pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF) and vigorously rocked for 15 minutes at 4°C. The extracts were then spun for 5 minutes at 12,000 g in a microfuge and the supernatant was stored at –80°C.

2.3.4 Enzyme-linked immuno-assay

TransAM AP-1 p-cJun ELISA-based kit (ActiveMotif) was used for measuring binding of nuclear phosphorylated cJun protein to its oligonucleotide consensus sequence. This sequence was pre-adhered to the 96-well plate and nuclear extracts were exposed to the wells. Binding was then measured according to the manufacturer's instructions using a simple colorimetric assay.

2.3.5 RNA extraction

Total RNA was extracted using Trizol reagent. Briefly, after treatment, the cells were lysed with Trizol and incubated at room temperature for 5 minutes. 200 µL of chloroform was added per 1 mL Trizol used and the samples shaken vigorously by hand for 30 seconds, incubated on ice for 2-3 minutes, then centrifuged at 14,000 g for 15 minutes at 4°C. The top aqueous phase was removed, to which 0.5 mL of isopropyl alcohol per 1 mL Trizol used, was added. The samples were then incubated overnight at –20°C. Upon thawing, the samples were centrifuged at 14,000 g for 10 minutes at 4°C. The supernatant was removed and the pellet washed with 1 mL of 75% ethanol per 1 mL Trizol used. After vortexing, the samples were centrifuged at 12,000 x g for 5 minutes at 4°C and the supernatant removed. The pellet was dried carefully and dissolved in 30 µL

of TE. RNA purity was determined by measuring its optical density at 260 nm and 280 nm. RNA integrity was assessed by 1% agarose gel electrophoresis (0.5 g agarose, 5 mLs 10x MOPS, 9 mLs formaldehyde, 2 uL of EtBr, and 26 mLs H_2O). Briefly, 3 uL of each RNA sample was combined with 2 uL of 10x MOPS (200 mM MOPS, 50 mM Sodium Acetate, 10 mM EDTA), 3.5 uL of 37% formaldehyde, and 10 uL of deionized formamide. The samples were heated at 55°C for 15 minutes and cooled on ice. Three uL of loading dye was added and the samples run in 1x MOPS buffer for approximately 2 hours at 100 V. The gel was soaked overnight in ddH_20 and visualized under UV light.

2.3.6 Reverse transcriptase reactions

After quantitation by spectrophotometer, 100 ng of RNA, diluted with sterile water and heated at 65°C for 5 minutes then placed on ice, was reverse transcribed to cDNA using PE Applied Biosystems reagents. The RNA samples were mixed with 1x RT buffer, 5.5 mM $MgCl_2$, 500 µM of each dNTP, 2.5 µM Random Hexamers, and either 1.25 U/µL Reverse Transcriptase or sterile water (for negative controls) to a final volume of 30 µL. The mixture was incubated for 10 minutes at room temperature, 45 minutes at 48°C, then 5 minutes at 95°C.

2.3.7 Real-time polymerase chain reaction

cDNA samples were mixed with PCR reagents (PE Applied Biosystems) and PCR amplification was performed on a Biorad iCycler. Data was recorded and analysed using Biorad iCycler software. The control gene used was hypoxanthine guanine phosphoribosyltransferase (HPRT), which is a housekeeping gene involved in purine scavenging. It was chosen as the control gene because HPRT was found to be in comparable concentration in the OTR in our samples. Each reaction contained: 2 uL of cDNA, 10x SYBR green buffer, 3 mM $MgCl_2$, 0.2 µM primers, 0.01 U/µL AmpErase, 0.025 U/µL AmpliTaq, 1 mM of dNTP mix with UTP, and sterilized water to a final volume of 50 µL. The reactions were carried out in triplicate and underwent 36 PCR

cycles, consisting of a denaturing phase at 95°C for 30 seconds, and an annealing and extension phase at 65°C for 60 seconds. These cycles were preceded by 2 minutes at 50°C and 10 minutes at 95°C. The former served to activate the AmpErase which cleaved any contaminating PCR products, while the latter deactivated the AmpErase and activated the AmpliTaq. The primers used are shown in Table 2 below. The threshold cycle of each well was recorded by the iCycler software and relative quantification of OTR was calculated using the mathematical 'Delta-delta' model presented by PE Applied Biosystems.

During real-time PCR, the threshold cycle (Tc, the cycle during which the fluorescence level rises significantly above the background fluorescence) of each sample is representative of the relative mRNA level of the gene of interest. Here, the OTR and HPRT genes were used and their relative expression ratio, *R*, was determined using the following equation:

$$R = 2^{-\{[Tc_{HPRT} - Tc_{OTR}]_{sample}\} - \{[Tc_{HPRT} - Tc_{OTR}]_{calibrator}\}}$$

$$R = 2^{-[\Delta Tc_{sample}] - [\Delta Tc_{calibrator}]}$$

$$(1) \quad R = 2^{-[\Delta\Delta Tc]}$$

This mathematical model, described in more detail by Pfaffl,[184] makes use of a "calibrator" sample which serves as a physiological reference on every PCR plate. Here, the no treatment control sample was used as a calibrator. This method eliminates the use of a standard curve, but only if the *efficiency*, E, of each reaction set (for both the gene of interest and for the control gene) are equal to within 10%. Reaction efficiency is ideal when at 100%, for this is where in each cycle of exponential amplification, the template doubles. This is illustrated in Table 3 below. Several factors can affect efficiency, such as the amplicon length, secondary structures, G/C content of the primers, and reagent concentrations. To confirm that this model was feasible to analyse my data, I determined that the OTR and HPRT primer efficiencies were equal to within 10%. To do this, I performed real-time PCR for OTR and HPRT on a standard curve dilution obtained

from a representative cDNA sample. Efficiency was calculated from the slope of the standard curve as follows:

$$(2) \quad E = 10^{[-1/\text{slope}]}$$

The amplification curves for OTR and HPRT can be seen in Figure 12a and b, and their respective standard curves in Figure 13a and b. Their efficiencies were calculated in Figure 13c and were determined to be: 97.4% and 101.1%, respectively. This way, the standard curve could then be bypassed and equation (1), or the 'Delta-delta' method of calculation was used to analyse data output.[185]

2.3.8 Radio-ligand binding assays

ULTR cells were grown to confluence in 12-well plates and washed two times with binding buffer (PBS with 10 mM MgCl2 and 0.2 % BSA, pH 7.4). Radioactive [tyrosyl-2,6-^{3}H]-OT from New England Nuclear (NEN) was used. To assess total ^{3}H-OT binding (New England Nuclear, NEN), 400 µL of binding buffer and 100 µL ^{3}H-OT (2.5 pmol ^{3}H-OT in binding buffer to a final concentration of 5 nM) was added to each well. This ^{3}H-OT concentration was used in order to saturate the oxytocin receptors, the kD of which is approximately 1 nM. To determine non-specific binding, select wells were given 395 µL of binding buffer and 100 µL of ^{3}H-OT (to a final concentration of 5 nM) and 5 µL of cold OT (1 mM of cold OT to a final concentration of 10 uM). Wells were incubated for 1 hr at room temperature and washed 3 times with ice cold PBS. Cells were lysed with 0.25 mL of 1 N NaOH per well. Lysate was put in a 2 mL scintillation vial, to which 2 mL scintillation cocktail was added (Scintisafe Plus 50%). Radioactivity count in dpm was determined using the Beckman Scintillation System, LS 5000 TD. Specific binding was calculated by subtracting the non-specific binding from the total binding.

2.3.9 Immunofluorescence

The primary antibodies used for immunofluorescence were as follows: 1:500 p-cJun from Santa Cruz, 1:500 OTR from Dr. T. Kimura (Osaka Medical Centre for Cancer and Cardiovascular Diseases, Japan), and 1:500 caveolin (BD Transduction Labouratories). $1x10^5$ cells were grown overnight on 8-chamber slides (VWR). After treatment, cells were fixed with 3.7% formaldehyde and 0.5% Triton X100 in 1x PBS for 15 minutes. Cells were then washed with 1x PBS, exposed to blocking buffer (5% BSA in 1x PBS) for 30 minutes, and incubated with primary antibody for either 2 hours (for OTR and caveolin) or overnight (for p-cJun). After washing 3 times for 5 minutes each with 1x PBS, cells were exposed to the secondary antibody for 30 minutes in the dark (for p-cJun and OTR, 1:200, anti-mouse-FITC-conjugated Sigma, #F-5262 and for caveolin, 1:200, anti-rabbit-TRITC-conjugated from Sigma, #T-6778). Chambers were removed and ~60 uL DAPI fixation solution was spotted on the slide. Cover slips were secured with nail polish and the slide was left to dry in the dark for ~15 minutes. Slides were visualized under the microscope and photographs taken using the Spot Advanced imaging system.

2.3.10 Western immunoblotting

Total cellular protein was extracted with 100 µL/well of 1x SDS lysis buffer (62.5 mM Tris-Cl pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, and 0.01% w/v bromophenol blue) using a cell scraper. Samples were sonicated for 10 seconds and stored at -80°C until used. Twenty-five µl (equal volume per sample per time point and treatment) of protein was mixed with 5x sample buffer, boiled for 5 minutes, and run on a 10% denaturing polyacrylamide gel (5 mL of 30% acrylamide/0.8% bisacrylamide, 3.75 mLs of 4x Tris-Cl/SDS ph 8.8, 6.25 mL of H_2O, 50 uL of 10% ammonium persulfate, and 10 uL of TEMED) using 1x running buffer for approximately 2.5 hours. Eight µL of protein molecular weight marker (Invitrogen #10748010) was run on every gel. The gel was transferred to a nitrocellulose membrane at 100 V for approximately 70 min at 4°C in

transfer buffer. The membranes were then washed in approximately 25 mLs of blocking solution (5% skim milk powder in TBS-0.1%Tween) for 1 hour. The primary antibodies for immunoblotting were used as follows: overnight incubation at 4°C with 1:200 of phospho-cJun antibody (Cell Signaling #9261), or 1 hour incubation at room temperature with 1:800 of cFos antibody (Oncogene #OP17). Both antibodies were diluted in 2 mLs of TBS-0.1% Tween in 1% skim milk powder. After washing 3 times for 5 minutes, membranes were exposed for 1 hour (in 10 mLs of TBS-0.1%Tween in 1% skim milk powder) to the HRP-conjugated secondary antibody (for cJun, 1:3500 of goat anti-rabbit from Santa Cruz #sc-2054, or for cFos 1:5000 of anti-mouse from Santa Cruz #sc-2055). The membrane was then rinsed with TBS-0.15% Tween 10 times for 10 min and exposed using ECL chemiluminescence reagents (Amersham Biosciences). Membranes were exposed on Super RX Fuji Medical X-RAY film for approximately 30 minutes (for p-cJun) and 5 minutes (for cFos).

2.3.11 Adenoviral infection

Adenoviral constructs (AdcJun, AdcFos, AdtTA) were generously donated by Dr. Michael B. Stemerman from the University of California Riverside. They were constructed by inserting a cDNA fragment coding for the protein of interest into a pAdlox shuttle vector and recombined with E1- and E3-deleted ψ 5 viral DNA in CRE8 cells. Expression of cJun and cFos are driven by a 7x tet/minimal cytomegalovirus promoter, which are further under the control of a tetracycline-responsive transactivator (tTA). For this reason, each infection must be accompanied by the AdtTA vector which expressed the tTA to drive cJun and cFos expression. As a control, 0.1 ug/uL tetracycline was added to prevent the activity of the AdtTA, thereby preventing the expression of the AdcFos and AdcJun.

With assistance from the Adenoviral Core facility of the Perinatal Research Centre, the vectors were propagated to the following titres: 8.1×10^9 pfu/ml (AdGFP), 1.06×10^{10} pfu/ml (AdtTA), 3.2×10^{10}pfu/ml (AdcJun), and 5.15×10^8 pfu/ml (AdcFos). ULTR cells were exposed to a combined (1:1) viral multiplicity of infection (MOI, or pfu/cell) of 30

for 6 hours. The virus was then washed off and left for a time course of 24 to 72 hours to allow for gene expression. As a control to ensure infection was working, the cells were incubated with the AdGFP vector and fluorescent pictures taken after 24 hours (data not shown).

2.4 STATISTICS

ELISA data were analysed using a paired t-test. PCR data were analyzed using a t-test (at 24 hours), and also normalized and analyzed using a Kruskall Wallis ANOVA on ranks followed by a Dunn's (Bonferroni) post-hoc test. Binding assay data were normalized and analyzed using a Kruskall Wallis ANOVA on ranks followed by a Dunn's (Bonferroni) post-hoc test. All data were expressed ± SEM. Level of statistical significance was $p < 0.05$.

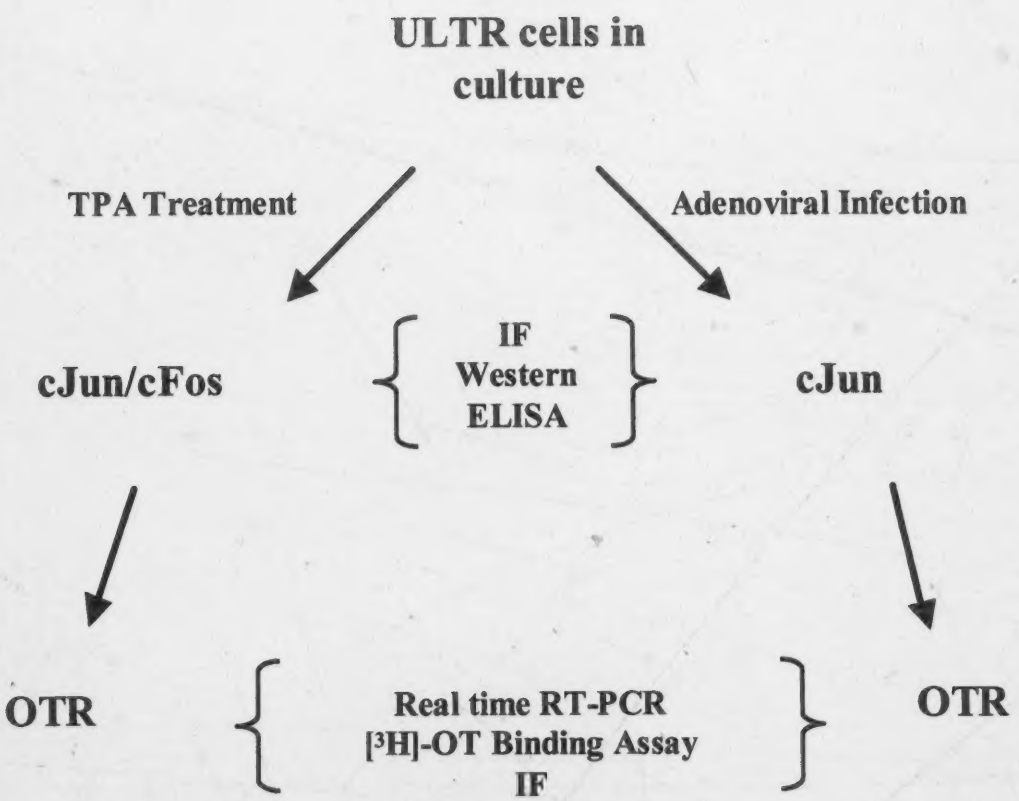


Figure 10. Experimental Design.

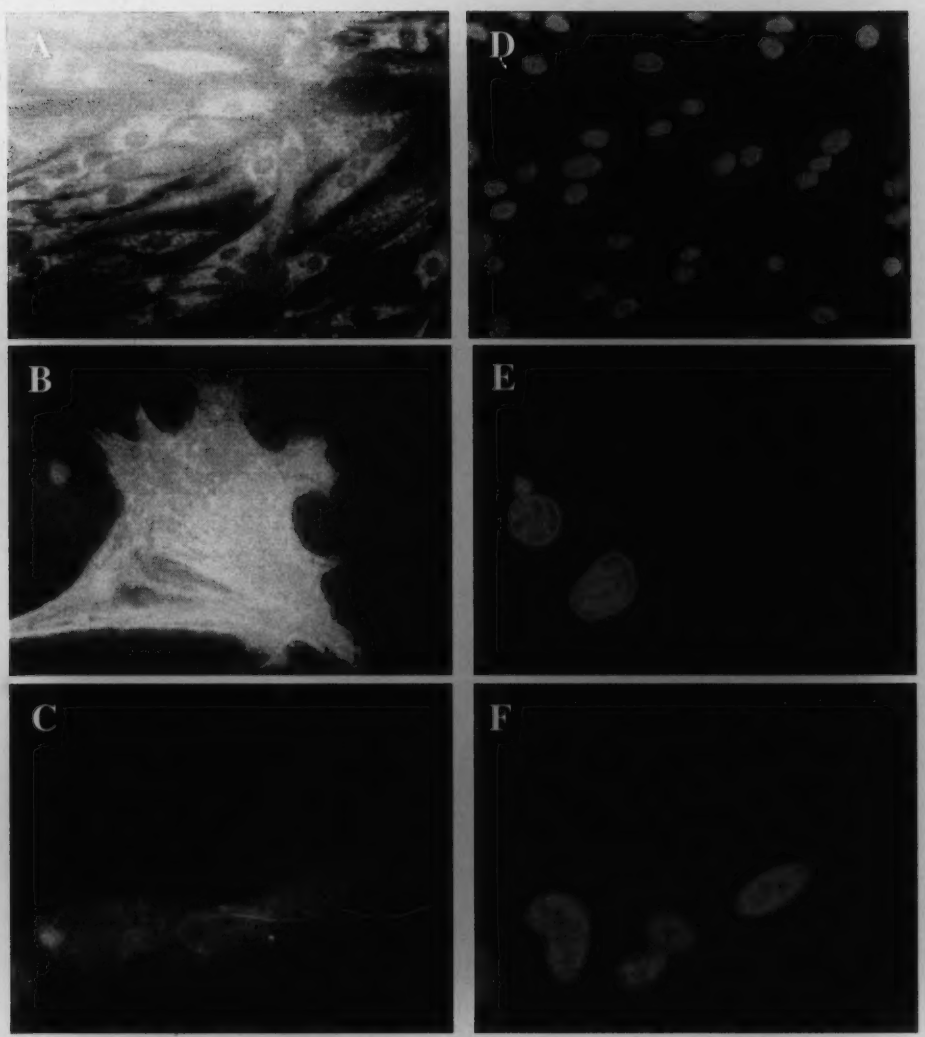


Figure 11. Immunofluorescent microscopy for smooth muscle actin in ULTR cells.

A) smooth muscle actin (40x), **B)** smooth muscle actin (100x), **C)** no primary antibody added. **D)** DAPI nuclear staining corresponding to **A)**, **E)** DAPI staining corresponding to **B)**, **F)** DAPI staining corresponding to **C)**. (Work done by Dr. Xin Fang, in the labouratory of BF Mitchell).

GENE	PRIMER SET	AMPLICON SIZE
Oxytocin Receptor (OTR)	Sense: 5'-GTACCCATCCAGCGACCAG-3' Antisense: 5'-GCGAACCTAAAGTTGACTCCC-3'	187
Hypoxanthine Guanine Phosphoribosyltransferase (HPRT)	Antisense: 5'-AGTCTGGCTTATATCCAACACTTCG-3' Sense: 5'-GACTTTGCTTTCCTTGGTCAGG-3'	101

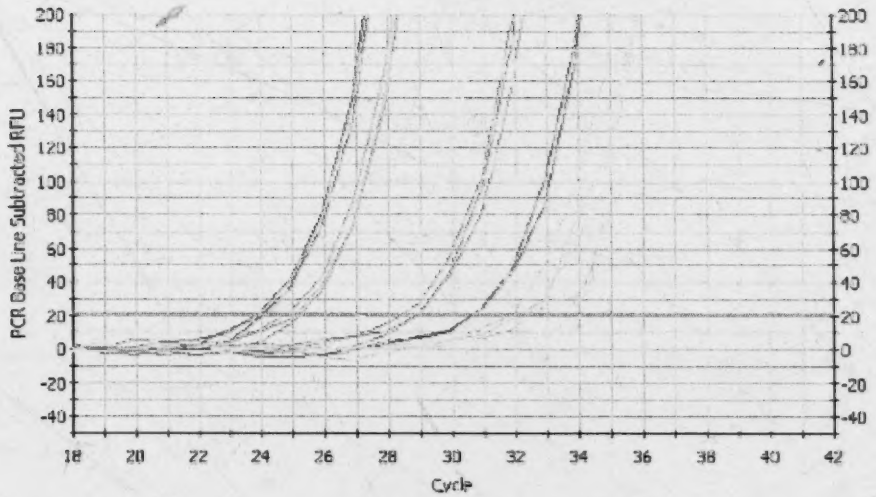
Table 2. Primer sets used for quantitative real-time RT PCR for OTR and HPRT.

The primers used for OTR were designed in our labouratory, while the sequences for the HPRT primers were provided by the labouratory of Dr. P. Halloran (University of Alberta)

Slope	Amplification	Efficiency
-3.55	1.9129	0.9129 (91.29%)
-3.50	1.9307	0.9307 (93.07%)
-3.45	1.9492	0.9492 (94.92%)
-3.35	1.9684	0.9684 (96.84%)
-3.30	2.0092	1.0092 (100.92%)
-3.25	2.0309	1.0309 (103.09%)
-3.20	2.0535	1.0535 (105.35%)
-3.15	2.0771	1.0771 (107.71%)
-3.10	2.1017	1.1017 (110.17%)

Table 3. This table demonstrates the efficiency of a standard curve as a function of its slope ($E = 10^{[-1/slope]}$). Optimal PCR efficiency (highlighted) is obtained with a slope of -3.30. Adapted from Stratagene.[186]

A)



B)

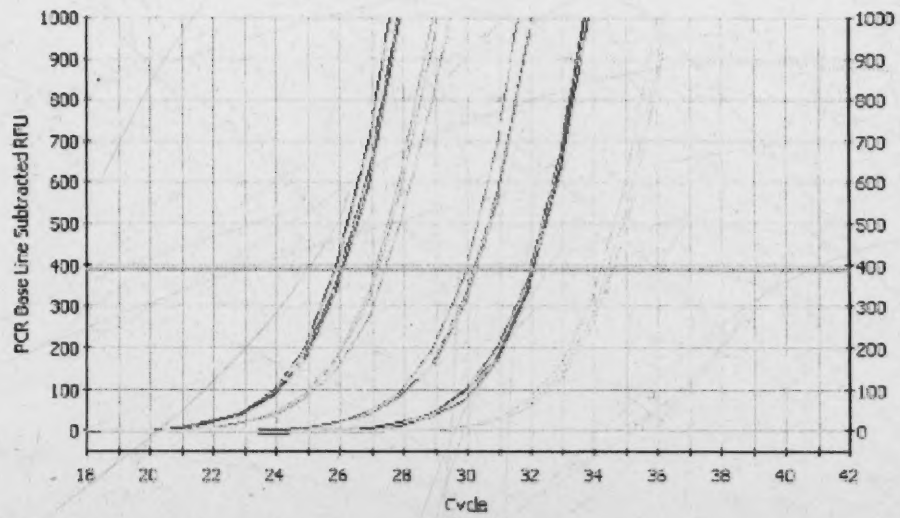
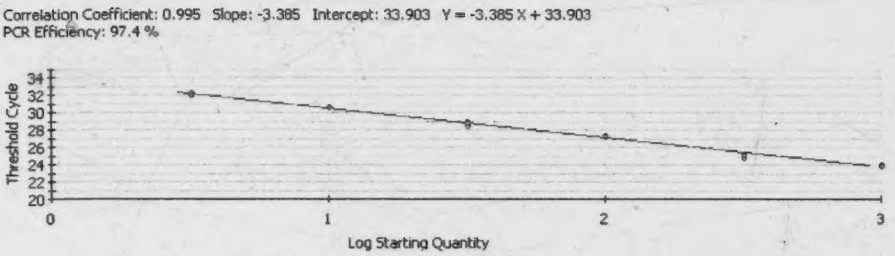


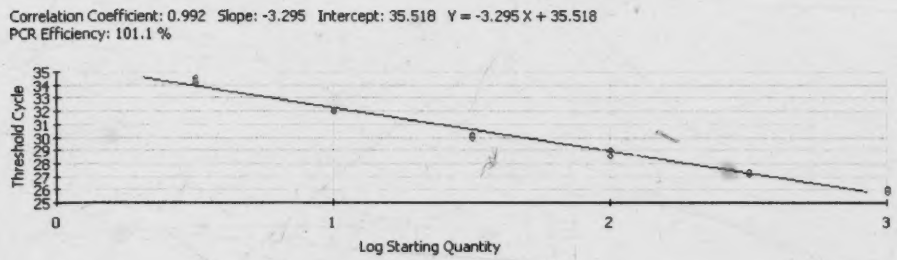
Figure 12. Amplification curves showing the threshold cycles ( ) of A) OTR mRNA, and B) HPRT mRNA in a standard curve dilution series of a representative ULTR sample, as determined by quantitative real-time RT-PCR.

■ 10^3 ■ $10^{2.5}$ 10^2 ■ $10^{1.5}$ ■ 10^1 ■ $10^{0.5}$

A)



B)



C)

$E_{OTR} = 10^{[-1/\text{slope}]}$ $E = 10^{[-1/-3.385]}$ $E = 1.974 = \mathbf{97.4\%}$

$E_{HPRT} = 10^{[-1/\text{slope}]}$ $E = 10^{[-1/-3.295]}$ $E = 2.011 = \mathbf{101.1\%}$

The efficiencies of the OTR and HPRT reactions are within 10%.

Figure 13. Standard curve threshold cycle as a function of the log of the starting quantity (in ng) of ULTR control samples. A) OTR cDNA amplified, B) HPRT cDNA amplified. C) Efficiency (*E*) calculation.

A standard curve is only used to determine reaction efficiency (*E*). The slope of the curves for OTR and HPRT above are used to calculate *E* which is seen in C). Once *E* for each gene is determined to be within 10% of one other, the standard curve is no longer used. See text for more details.

CHAPTER 3. RESULTS.

3.1 AIM ONE

3.1.1 TPA treatment increases cJun and cFos in ULTR cells

To confirm that the AP-1 was being activated by TPA treatment, ULTR cells were treated for a time course of 15 minutes to 2 hours with TPA, as described above. Phosphorylated (p)-cJun expression increased significantly at 30 minutes. This was demonstrated by immunofluorescence (Figure 14) and western immunoblotting (Figure 14a). cFos increased significantly at 15 minutes as demonstrated by western immunoblot (Figure 15b).

3.1.2 TPA treatment increases AP-1 activity in ULTR cells

While the levels of cFos and cJun proteins increased, any change in function had yet to be assessed. To see if the increase in cFos and cJun translated to an increase in AP-1 activity (as assessed by binding to its consensus sequence), an ELISA-based assay was performed. After 30 minutes of TPA treatment, AP-1 binding, as represented by detection of phosphorylated cJun bound to the TRE consensus sequence, increased significantly by almost 6-fold (Control: 0.32 OD/ug $\pm$ 0.07; TPA: 1.88 OD/ug $\pm$ 0.54) ($n=4$, $p < 0.05$) (Figure 16).

3.1.3 TPA treatment increases OTR mRNA in ULTR after 24 hours

After confirming the increase in AP-1 levels and activity in response to TPA treatment, OTR mRNA levels were measured by quantitative real-time PCR over a 24 hour period. Expressed as a ratio to the calibrator sample (see section 2.3.6 for details), OTR mRNA after 1 hour of TPA treatment was 1.60 $\pm$ 0.34 and after 24 hours of treatment was 2.58 $\pm$ 0.44 ($n=5$, $p < 0.05$) (Figure 17). The control levels did not change.

3.1.4 Effect of TPA treatment on [^{3}H]OT- binding

To assess OTR function, a radio-ligand binding assay was performed. After normalizing the data, each timepoint was expressed as a ratio to its own control. After 1 hour of TPA treatment [^{3}H]OT binding rose 2.23 ± 0.23 times over control levels, and after 24 hours of treatment binding was 0.903 ± 0.098 times control ($n=6$, $p < 0.02$) (Figure 18).

3.1.5 Localization of OTR and caveolin

To examine the localization of OTR in relation to the plasma membrane, OTR was detected by immunofluorescence and co-stained with caveolin (used as a membrane marker) with and without TPA treatment for 24 hours (Figure 19). Qualitatively, no change could be detected using this technique.

3.2 AIM TWO

3.2.1 Adenoviral infection of GFP

ULTR cells were infected with a titration of adenoviral vector for GFP (AdGFP). This was done both to obtain the optimal MOI for subsequent infection and also to ensure the Adenoviral protocol would work in ULTR cells. The MOI at which the cells showed optimal GFP without significant cytotoxicity (as assessed by visual inspection) was 30 pfu/cell, and GFP was found to overexpress within 24 hours of exposure to the cells (data not shown).

3.2.2 Adenoviral infection of AdcJun increases cJun in ULTR cells

ULTR cells were infected with the adenoviral vectors AdcJun and AdTet. To confirm that the cJun was being overexpressed following adenoviral infection, western immunoblotting and immunofluorescence was performed. The western blot showed that cJun expression increased significantly by 24 hours after infection but rose even further

by 48 hours (n=3) (Figure 20) This level was sustained at 72 hours. These results were confirmed by immunofluorescence, which confirmed the increase in cJun after 48 hours of infection (Figure 21).

3.2.3 Adenoviral overexpression of cJun does not affect OTR mRNA

After confirmation of adenoviral cJun overexpression, cells were infected as above, and OTR mRNA was measured over a time course of 0, 48, and 72 hours. Results demonstrate that cJun overexpression did not affect the expression of OTR mRNA (Figure 22).

3.3 SUMMARY

In summary, these data demonstrate that cFos and phosphorylated cJun increased in protein expression, and that AP-1 increased binding to the TRE consensus sequence in response to TPA treatment. Further, OTR responded in two different ways. First, OTR mRNA increased significantly at 24 hours in response to TPA, while the oxytocin binding profile showed an increase at 1 hour followed by a return to control levels at 24 hours.

In response to adenoviral infection with AdcJun, cJun protein was successfully overexpressed, as shown by immunofluorescence and western blotting. However, OTR mRNA levels were not affected by cJun overexpression as measured by quantitative real-time RT-PCR.

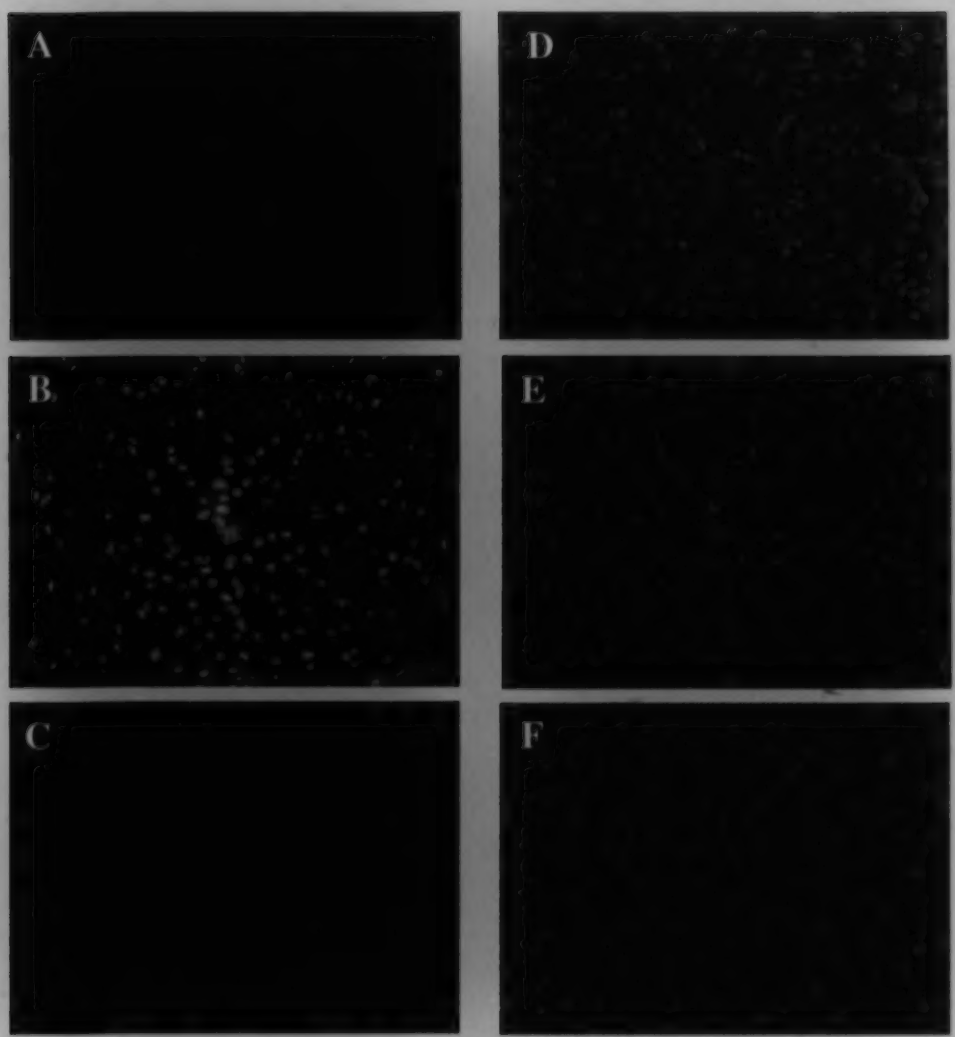


Figure 14. Immunofluorsecent microscopy demonstrating the effect of TPA treatment on p-cJun expression in ULTR cells (20x).

ULTR cells were treated with TPA (100 ng/mL) for 0.5 hours and stained for p-cJun. **A)** no treatment. **B)** TPA treatment, demonstrating an increase in p-cJun. **C)** Treated, but not incubated with primary antibody. To assess cell viability and density, nuclear DNA was visualized using DAPI. **D)** no treatment. **E)** TPA treatment. **F)** No primary antibody.

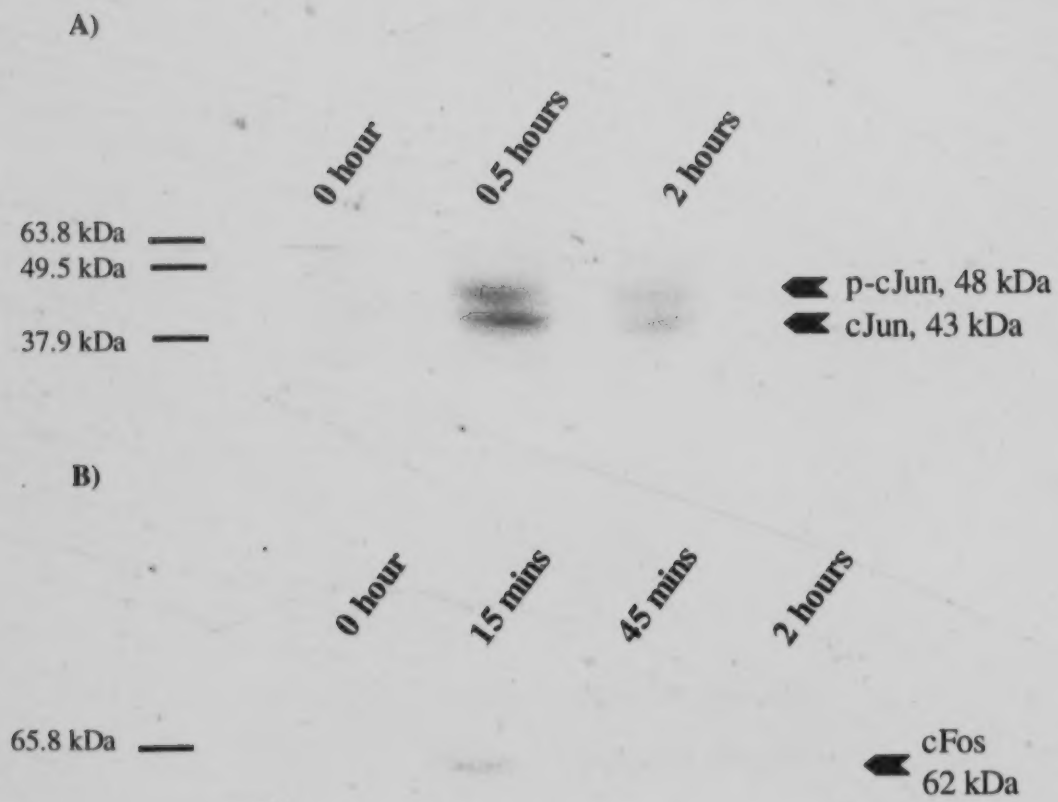


Figure 15. Western immunoblots demonstrating the effect of TPA treatment (100 ng/mL) on p-cJun and cFos expression in ULTR cells.

ULTR cells were treated with TPA. Protein extracts were run on acrylamide gels and immunoblotted as described in Materials and Methods. Representative immunoblots for p-cJun (**A**), and cFos (**B**) are shown above and demonstrate that TPA causes short-term induction of both AP-1 proteins. Two hours after treatment, levels of both cJun and cFos are returning to control levels.

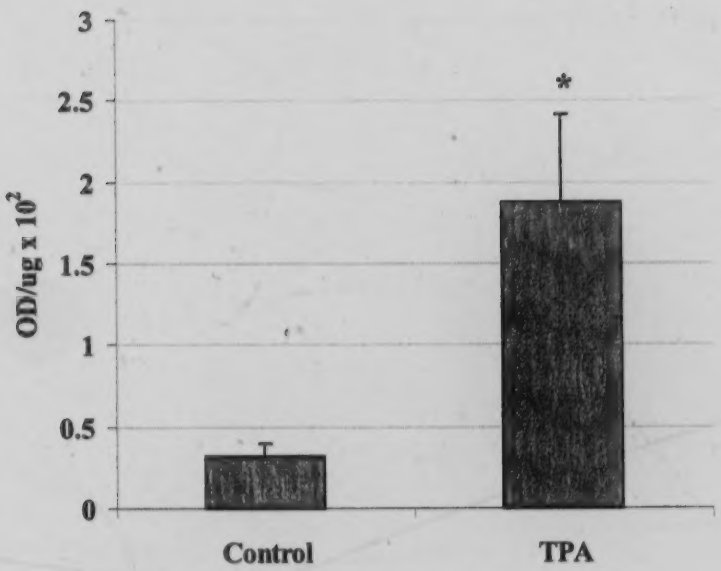


Figure 16. Effect of TPA treatment (100 ng/mL) on binding of p-cJun the TRE consensus sequence, as demonstrated by an ELISA-based assay.

After TPA treatment 0.5 hours, an ELISA-based assay was performed using nuclear extracts, as described in Materials and Methods. Colorimetric readings (measured in OD, or optical density) represent phosphorylated cJun, as part of the AP-1 dimer, binding to the TRE consensus sequence. TPA treatment increased binding almost 6-fold compared to control levels. ($n=4$, $p < 0.05$)

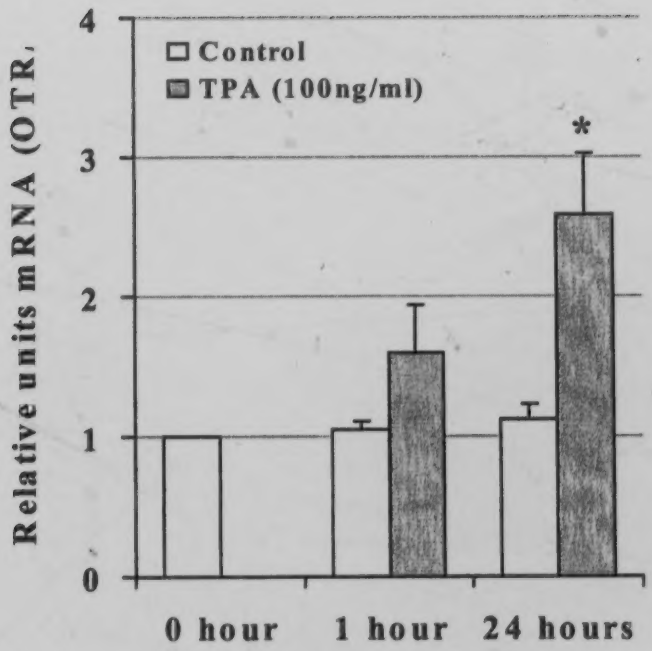


Figure 17. Effect of TPA treatment (100 ng/mL) on OTR mRNA expression.

After TPA treatment, OTR mRNA was measured by real-time quantitative RT-PCR as described in Materials and Methods. OTR mRNA expression increased significantly after 24 hours. The control samples did not change. Expressed as a ratio to the calibrator sample (see text for details). ($n=5$, $p< 0.05$)

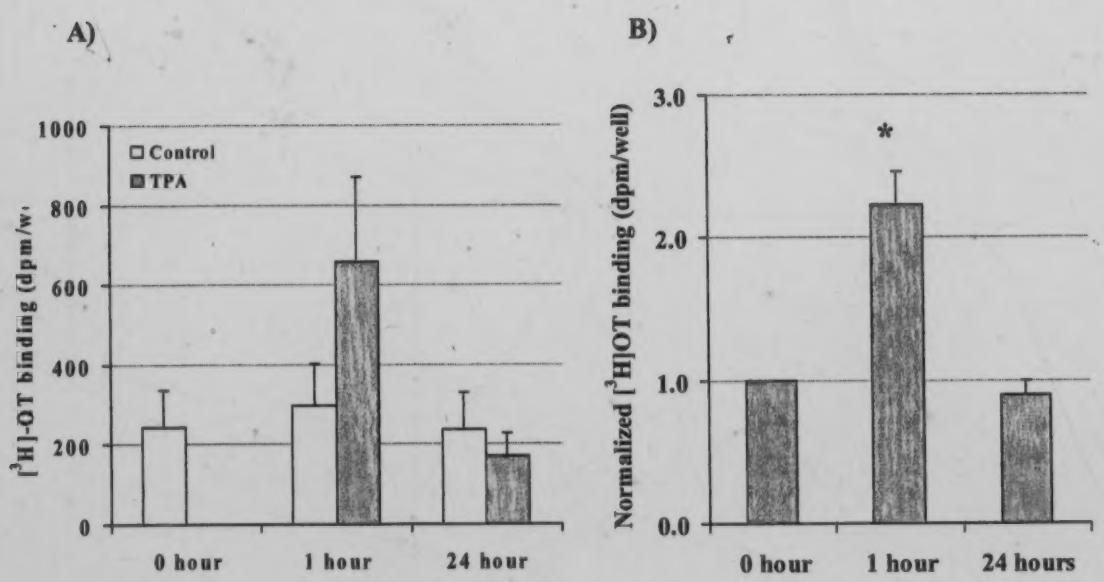


Figure 18. Effect of TPA treatment (100 ng/mL) on [^{3}H]OT binding in ULTR cells.

Cultured ULTR cells were treated with TPA and [^{3}H]OT binding assessed as described in Materials and Methods. [^{3}H]-OT binding increased significantly after 1 hour of TPA treatment and returned to control levels by 24 hours. **A)** Raw data (dpm/well) **B)** Data normalized for control values. ($n=6$, $p < 0.05$)

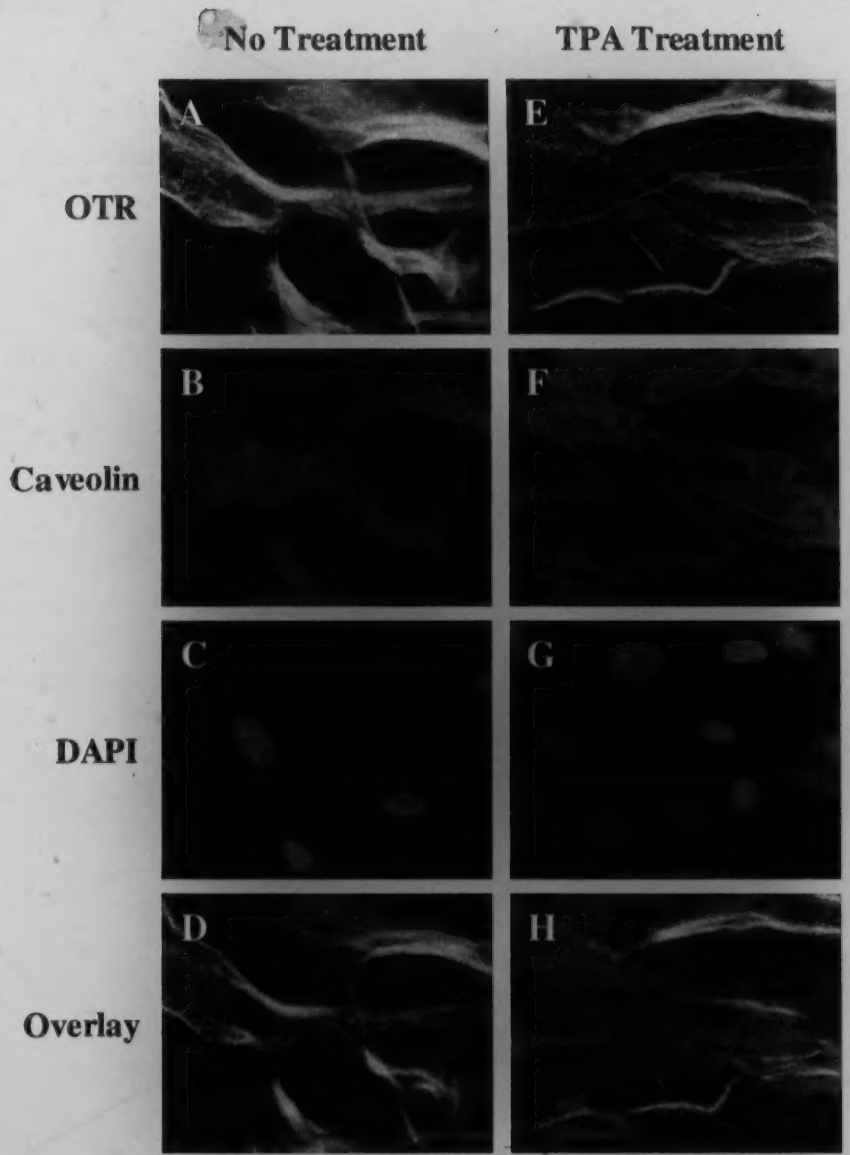


Figure 19. Immunofluorescent microscopy demonstrating the effect of TPA treatment (100 ng/mL) on localization of OTR and Caveolin (100x).

ULTR cells were treated with TPA for 24 hours and stained for OTR, caveolin, and DAPI. **A-D)** no treatment; **E-H)** TPA treatment. **A, E)** OTR; **B, F)** caveolin; **C,G)** DAPI; **D,H)** overlay. Note: control pictures with no primary antibody incubation are not shown.

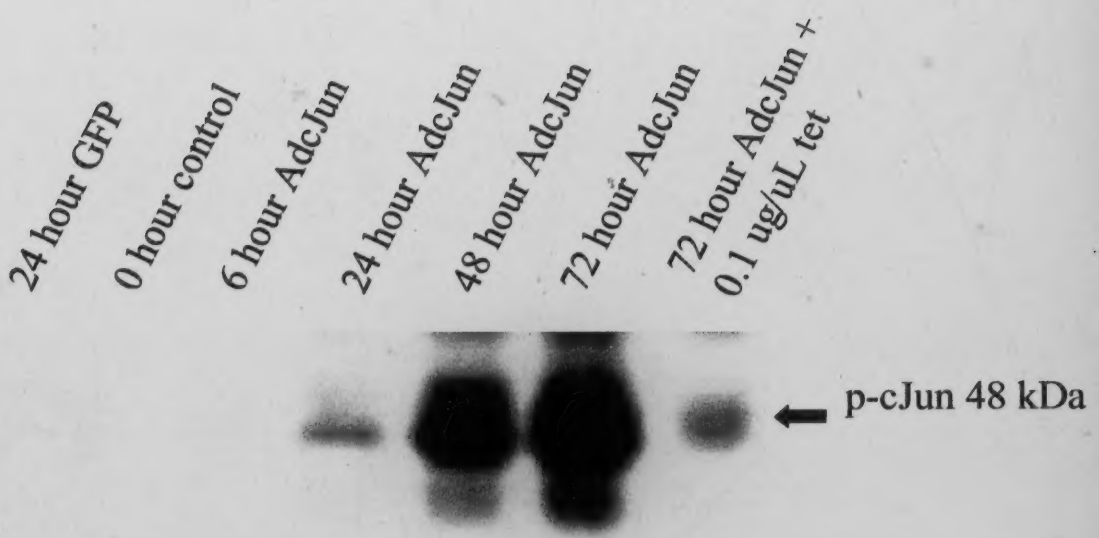


Figure 20. Western immunoblot demonstrating the effect of AdcJun adenoviral infection in ULTR cells.

cJun increases in response to AdcJun infection. This increase is partially inhibited by the addition of tetracycline (last lane).

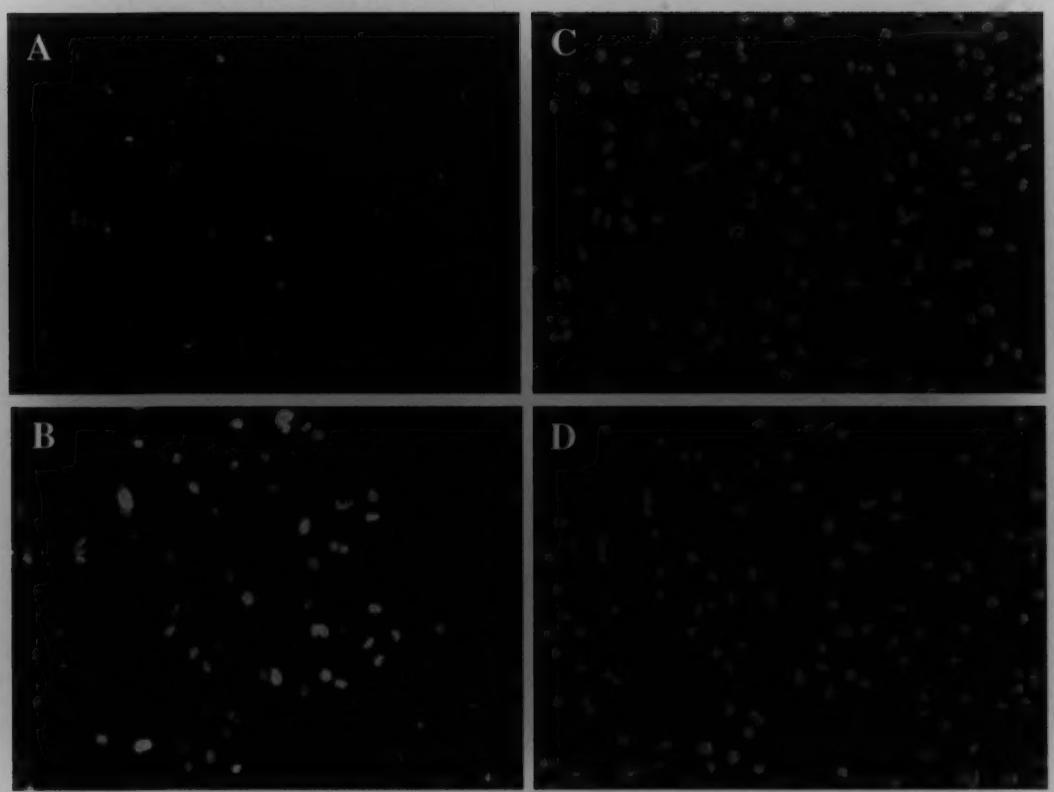


Figure 21. Immunofluorescent microscopy demonstrating the effect of AdcJun adenoviral infection on p-cJun expression in ULTR cells.

ULTR cells were incubated with AdcJun adenoviral vector for 48 hours and stained for p-cJun. **A)** no treatment; **B)** AdcJun infection. The latter shows an increase in p-cJun at 48 hours. To assess cell viability and density, nuclear DNA was visualized using DAPI. **C,D)** corresponding DAPI stain. Note: control pictures with no primary antibody incubation are not shown.

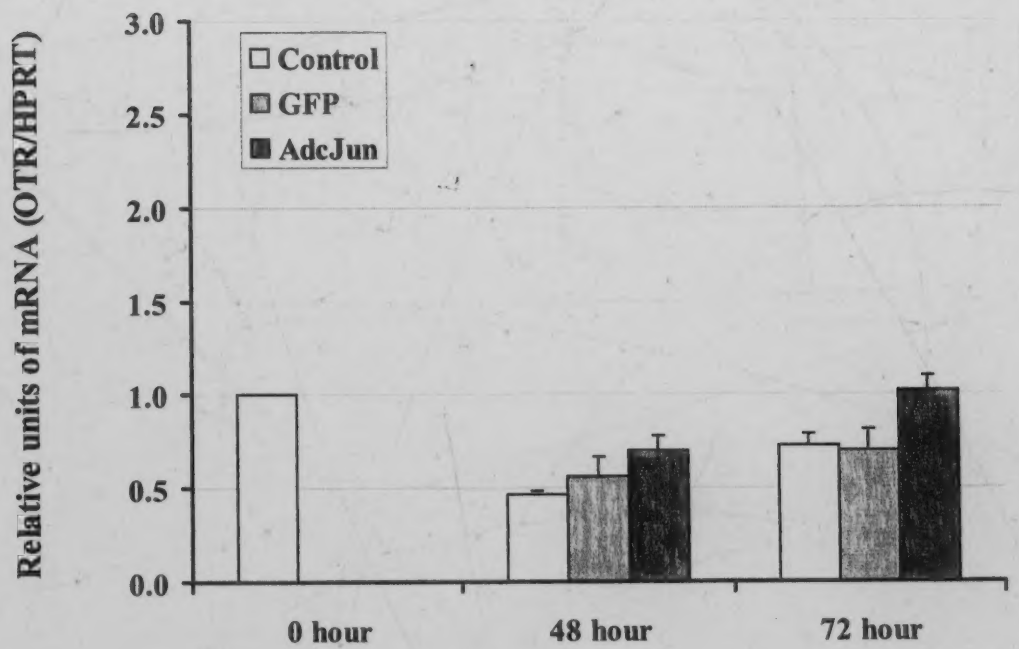


Figure 22. Effect of adenoviral infection of AdcJun on OTR mRNA expression.

After AdcJun infection, OTR mRNA was measured by quantitative real-time RT-PCR as described in Materials and Methods. OTR mRNA did not change in response to cJun overexpression. Expressed as a ratio to the calibrator sample (see text), OTR mRNA did not change significantly after cJun overexpression. ($n=3$, $p > 0.05$)

CHAPTER 4. Discussion and Conclusion.

4.1 DISCUSSION

It is well-established that that the OT/OTR system plays an important part of the parturition process, specifically in mediating strong uterine contractions. Whether this is an essential system for parturition to occur remains to be determined. However, it is known that the human and rat myometrial concentration of OTR increases dramatically at term.[54, 103] The exact mechanism of this upregulation is poorly understood though clues may be found in the promoter region of the OTR gene. The promoter region contains many putative binding sites for regulatory transcription factors, and at this level, regulation is complex and multifactorial. For research purposes, the transcriptional machinery must be broken down into feasible parts, and as such, one typically examines one or two transcription factors at a time. For this project, the focus was on the transcription factor AP-1. Concentrations of the AP-1 proteins have previously been shown to increase at term in rat myometrium,[160] and because several CAP genes contain consensus TREs this suggests that AP-1 may be a common mediator of CAP gene induction. Indeed, this increase in myometrial AP-1 occurs prior to the increase in both OTR and Cx-43.[164, 165] Geimonen et al found that Cx-43 in human myocytes was responsive to PKC through TPA stimulation and that this effect was dependent on a specific AP-1 binding site.[166] The role of AP-1 on Cx-43 regulation is well-established, but studies of AP-1 regulating human OTR transcription are few. Bale et al have demonstrated that the rat OTR gene is highly responsive to TPA treatment, and even more so when co-treated with forskolin.[167] The promoter region of the rat OTR gene is organized quite differently than that of the human and also contains three, rather than two, AP-1 binding sites. However, the same group did demonstrate in MCF7 human breast cancer cells that TPA treatment caused a forty-fold increase in binding of ^{125}I-labeled OTA after 24 hours.[167] For these reasons, this study of AP-1 on human OTR transcription in myometrial cells was performed.

The results presented here demonstrate that there are several mechanisms at play in the regulation of OTR by PKC-mediated pathways - both genomic and non-genomic, and that AP-1 may in fact not be the principle factor involved. The first aim of this study used a pharmacological approach by stimulating ULTR cells with TPA. The first objective was to confirm the increase in AP-1 (represented by cJun and cFos detection) in response to TPA. This increase was demonstrated by using immunofluorescence and western immunoblotting (Figures 14 & 15). Since increase in protein does not necessarily reflect an increase in function, an ELISA-based assay was performed to ensure the increase in AP-1 due to TPA treatment was increasing its binding to the TRE consensus sequence (Figure 16).

In the experiments examining the effect of TPA treatment on OTR, we found that TPA treatment, presumably working through PKC-mediated AP-1 activation, significantly increased the expression of OTR mRNA after 24 hours by approximately 2.5 times (Figure 17). When considering the findings of other groups, there appears to be large variation in the estimated increase of OTR. Estimates range from a 180-fold increase in OT-binding[103] to a 27-fold increase in rat OTR mRNA from the beginning of pregnancy to parturition, most of which occurring in the 24 hours preceding delivery.[102] Likewise, Kimura et al found a 300-fold increase in mRNA expression (as determined by Northern blotting) in labouring versus non-pregnant myometrium.[104] The latter estimate may be high, for receptor binding data from Fuchs et al shows that there is an approximate 5-fold increase in OT-binding from *non*-pregnant versus *early*-pregnant myometrium alone. Considering the various techniques and experimental models used to study the rise in myometrial OTR, a certain inconsistency is expected. In addition, it has recently been demonstrated that there is a spatial variance of OTR expression in different areas of the uterus, and that OTR upregulation is not uniform among all myometrial cells.[103] Regardless, the 2.5-fold induction seen here is statistically significant, and is the first known study to make use of the highly sensitive, novel technique of quantitative real-time RT-PCR. These data suggest that PKC stimulation contributes to OTR induction and that this is correlated with an increase in AP-1.

Physiologically relevant PKC-stimulating factors may include growth factors, some cytokines, and even OT itself. In fact, we propose that OT binding regulates the production of OTR through a positive feedback loop, causing the OT/OTR system to perpetuate its own "feed-forward" mechanism (Figure 8). In this way, OT-induced PKC activation may drive OTR transcription. This could occur via AP-1, as we have suggested in this study, or through other PKC-mediated transcription factors. Both NFkB and NF-IL6 are potential candidates, for they both have potential binding sites in the OTR promoter region.[90, 124] Therefore, to identify whether the OTR mRNA induction by TPA was in fact due to AP-1 versus an alternate PKC-mediated system, adenoviral overexpression was used.

The second phase of this study used adenoviral infection technology to overexpress the AP-1 protein cJun in myometrial ULTR cells. This technique provides a direct method to increase the expression of a given gene. In this way, we were able to observe whether the effect seen in the first phase of the project was due directly to AP-1. cJun was chosen to be overexpressed in the cells because unlike cFos it is capable of forming homodimers. cJun overexpression was successful, in that it dramatically increased cJun expression (Figures 20 & 21). Functional changes (i.e. TRE binding) were not examined. Interestingly, cJun overexpression did not alter OTR mRNA expression (Figure 22). This suggests that either AP-1 is not involved in OTR induction or, there is an alternate AP-1 dimer besides the cJun:cJun dimer that is responsible. As mentioned above, AP-1 is made up of a dimer of proteins from members of the Jun and Fos family and different dimer combinations have different binding affinities for the TRE.[173] Some even loosely bind the CRE (cAMP response element), of which there is one in the proximal region of the OTR promoter. Interestingly, Mitchell et al have found that the expression profiles of the various Fos and Jun proteins differ at term in the rat uterus,[165] which may indicate that various dimer combinations differ in regulating gene expression.

Aspects of both genomic and non-genomic regulation by PKC were observed in this study. In contrast to what was expected, the binding assays showed a spike of [^{3}H]-OT binding at 1 hour and a return to control levels by 24 hours. This indicates that there is an initial increase in OTR binding capacity, likely independent of genomic activity,

occurring at the membrane level. By 24 hours, this effect had reversed. Possible mechanisms of this reversal include receptor internalization and/or receptor desensization through a conformational change. We propose that the changing OT-binding profile is more representative of the sensitivity of the receptor than actual protein levels. Previous studies on the desensitization of OTR suggest that phosphorylation on its cytosolic face induces conformational changes that alter its ligand binding capacity. Plested et al have identified several kinases that have potential docking/phosphorylation sites on the OTR, such as PKC, PKA, GRKs, CaM IIK, and PKG.[149] Interestingly, the GRKs have been identified as mediators of arrestin binding. As mentioned, arrestins uncouple GPCRs from their G proteins and target the receptors for internalization. In addition, PKC has also been shown to associate with OTR and may induce conformational changes that lead to a change in receptor sensitivity.[150] To date, we have not been able to find any studies that demonstrate that the direct association of any of these kinases with OTR leads to an *increase* in OT-binding. However, a study by Strosser et al demonstrated PKC stimulation (through TPA) increased OT-binding in rat hypothalamic neurons.[136] It remains to be determined if this occurs through direct association of PKC and OTR. However, this effect could be prevented by pre-treatment with a PKC inhibitor. Interestingly, the same study found a *decrease* in OT-binding after PKC stimulation in rat astrocytes. Evidently, cell- and tissue-specific context is important when studying the OT/OTR system. We propose that our data, showing an increase in PKC-mediated OT-binding after 1 hour of TPA treatment, reflects a cell-specific response and is regulated in part by the phosphorylation state of OTR. Further studies are needed to address this issue.

In order to address whether the changing OT-binding profile is due to membrane receptor internalization, cells were stained for OTR and caveolin (used as a membrane marker) after 24 hours of TPA treatment. Whether OTR localization was affected is difficult to ascertain using this technique. Confocal microscopy would be useful to address this issue. Further, cells should be examined after 1 hour of TPA treatment to see if there is a change in OTR localization to the membrane.

4.2 PROJECT LIMITATIONS

This study was undertaken in a cell culture system, using the ULTR immortalized myometrial cell line as a model. There are limitations in using both a cell culture system and an immortalized cell line. Cultured cells are removed from the context of the tissue and as such, are independent from interactions with other cells that may affect their function. This may result in the cultured cells expressing an incomplete or compensatory phenotype that is not physiological for its cell type. Further, a cell line is genetically modified and is therefore not completely representative of the type of cell it represents. Compromises must be made in experimental designs in order to properly isolate a variable, or to make a study more feasible. A cell culture system is one way to reach such a physiological compromise.

The first aim of this study used a pharmacological approach to address the regulation of OTR by AP-1. With a pharmacological approach, there are typically non-specific effects of the agent being used. In this case, TPA was used, and it is classically recognized as an analogue of DAG. TPA is a potent stimulator of PKC therefore PKC remains perpetually in the active state until degraded. Longterm TPA treatment (i.e. 24 hours) therefore results in PKC downregulation. PKC is known to regulate many cellular processes in an isozyme- and cell-type-specific manner, such as differentiation, proliferation, secretion, gene expression, and smooth muscle contraction.[117, 187] This wide range of effects may confound the results discussed here. For example, the transcription factors NFkB and NF-IL6 are also responsive to PKC stimulation, and there is one NFkB and three NF-IL6 binding sites in the OTR promoter region. The stimulation of alternate intracellular processes must be taken into consideration when examining the results of this part of the study.

Just as a pharmacological approach has limitations, so does adenoviral infection. The use of a virus, though non-replicating, introduces a foreign element into the cell which may alter the phenotype of the cell by turning on an immune response. Further, the overexpression of the gene may be so intense so as to eclipse any physiological systems

which work in concert with the gene product. It is important to keep this in mind when using adenoviral technology.

4.3 FUTURE STUDIES

Many questions remain unanswered in developing the concepts presented here. Future studies to be conducted include:

- Confirm the stimulation of PKC after TPA treatment using a PKC activity assay.
- Repeat the TPA treatments in the presence of a PKC and/or AP-1 inhibitor to see if the effects seen to date an be inhibited using specific inhibitors.
- Optimize the adenoviral co-infection of cFos and cJun. Co-infection of cJun and cFos was attempted in one experiment the cFos expression did not increase. Before examining the effect of this co-infection on OTR expression, it will be necessary to optimize the infection assay to obtain an increase in cFos. This could be done either by altering the MOI or the incubation time of the virus.
- Immunofluorescent microscopy for OTR and caveolin after TPA treatment for 1 hour
- Perform the TPA treatments in the presence of E_2 (or coinfect adenoviral expression vectors for the AP-1 proteins and ER) to examine whether the AP-1/ER association affects OTR transcription.
- Pretreat cells with demethylating agent 5-azacytidine (Aza-C) before doing relevant treatments. This may allow better access of the transcription factors to their binding sites. The OTR gene is naturally demethylated at term but in vitro this may not be the case.
- It would be worthwhile to examine the binding profile of the OT/OTR system beyond 24 hours to see if the increase in OTR mRNA observed after 24 hours of TPA treatment translates to an increase in functional receptors.

4.4 CONCLUSION

The question posed in this study was whether AP-1 is a regulator of OTR gene expression in uterine muscle cells. The pharmacological experiments demonstrate that there are several mechanisms at play in the regulation of OTR by PKC-mediated pathways -- both genomic and non-genomic. The adenoviral study to date suggest that AP-1, at least in the dimerized form examined here, is not the principle transcription factor mediating the response seen by TPA treatment. The results presented here support a PKC-mediated 'feed-forward' mechanism of OTR regulation, but whether this effect is mediated by AP-1 warrants further study.

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